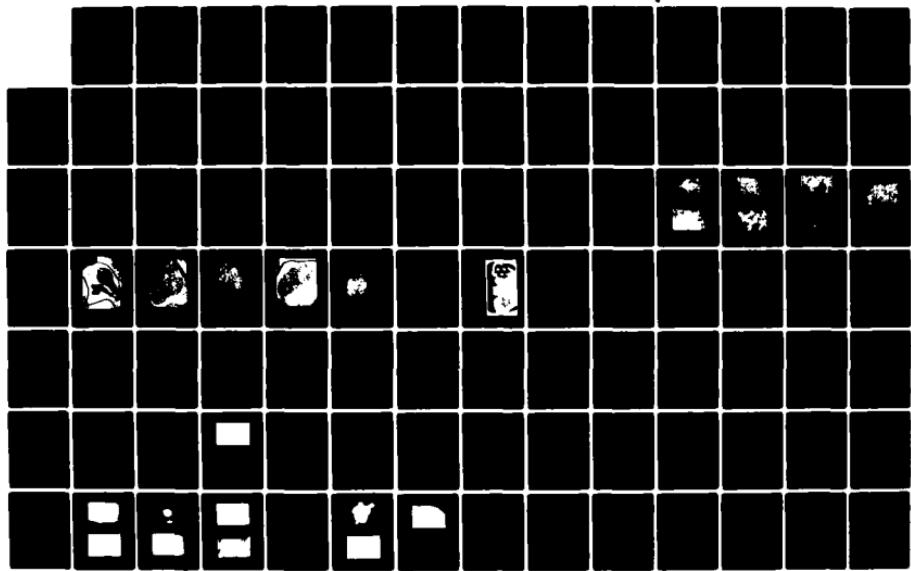
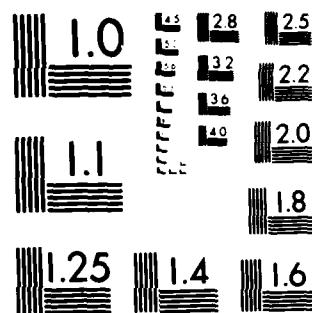


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REPORT NUMBER IX

CONTROL OF HEMOTROPIC DISEASES OF DOGS

Annual Progress Report

Miodrag Ristic

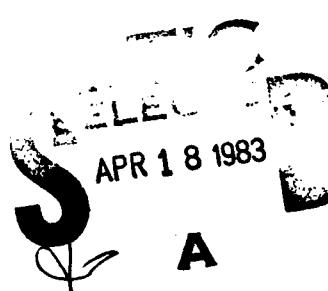
January 1, 1978 - December 31, 1978

Supported by

U.S. ARMY MEDICAL RESEARCH AND DEVELOPMENT COMMAND  
Washington, DC 20314

Contract No. DADA 17-70-C-0044

College of Veterinary Medicine  
University of Illinois  
Champaign-Urbana, Illinois 61801



Copies of this report will be provided to the appropriate U.S. Government agencies for inclusion in the Technical Abstract Bulletin and the Semi-Monthly Abstract Journal.

The findings in this report are not to be construed as an official Department of the Army position unless so designated by other authorized documents.

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FORWARD

In conducting the research described in this report, the investigators adhered to the "Guide for Laboratory Animal Facilities and Care," as promulgated by the Committee on the Guide for Laboratory Animal Resources, National Academy of Sciences - National Research Council.



A

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## I. SUMMARY OF PROGRESS DURING 1978

During the past year of support, answers to 3 research objectives have been sought and serodiagnostic services in support of control of blood diseases of military dogs in endemic areas performed. These objectives were (A) the effect of low level tetracycline treatment initiated during the chronic (carrier) phase of infection with Ehrlichia canis on the outcome of the disease; (B) Development of a method for in vitro cultivation of Babesia canis and use of culture-derived antigen as immunogen for canine babesiosis; (C) Study of relationship between causative agents of human sennetsu rickettsiosis and canine ehrlichiosis.

### A. The effect of low level tetracycline treatment initiated during the chronic (carrier) phase of the infection with E. canis on the outcome of the disease.

The United States Army Medical Research Unit in Kuala Lumpur, Malaysia, in collaboration with this laboratory, has undertaken a series of studies in an effort to obtain knowledge needed for formulating standard operational procedures (SOP) for control of canine ehrlichiosis or tropical canine pancytopenia (TCP). The first studies to this effect concerned application of low level tetracycline during the early phases of infection (please see Report VII, 1977). Studies of the past year concerned examination of the effect of low-level tetracycline on dogs chronically infected with E. canis.

Administration of low-level tetracycline 3 mg/lb/day per os was shown to be effective in freeing dogs from the chronic (carrier) E. canis infection. A slow but continuous decline in antibody titer using the indirect fluorescent antibody (IFA) test was indicative

of the efficacy of the drug to destroy residual infection in these dogs. As evidenced by back-challenge, chronically infected dogs freed from E. canis by tetracycline possessed a considerable degree of protective immunity. This immunity seems to be more efficient in protecting the animal from development of clinical diseases rather than reinfection.

B. Development of a method for *in vitro* cultivation of *Babesia canis* and use of culture-derived antigen as immunogen for canine babesiosis.

Contrary to canine ehrlichiosis, there are no effective therapeutic and chemoprophylactic methods for control of canine babesiosis in military dogs under field conditions. Studies of the mechanism of protective immunity with various Babesia species, however, showed that protection can be induced with inactivated immunogens. Thus, the aim of this study was to develop a cell culture method for production of B. canis antigens which may be used as immunogens against canine babesiosis.

Babesia canis derived from parasitemic dogs was propagated in primary cell culture in modified RPMI 1640. Relative numbers of extracellular and intracellular parasites were monitored daily. The maximum intracellular parasitemia occurred at 24 hours of cultivation. Extracellular Babesia forms reached maximal levels at 48 hours. Stages of erythrocytic invasion and different phases of replication of the organism were demonstrated by light and electron microscopy. Ultrastructural studies demonstrated the presence of a surface coat on the extracellular (merozoite) forms of B. canis.

Soluble Babesia antigens were isolated from the cultures by ammonium sulphate precipitation and molecular sieve chromatography

in Sephadex G-200. The antigens reacted specifically with immune serum obtained from dogs recovered from B. canis infection when tested in gel diffusion. Antigens derived from primary cell culture appeared to be heterogeneous but were all eluted in the first peak of Sephadex G-200 indicating a molecular weight around 900,000. Antigens obtained from serum of a dog suffering from acute babesiosis had limited heterogeneity and co-eluted with albumin corresponding to a molecular weight of 60,000 to 70,000. The antigenicity was destroyed by heating at 100° C for 15 min and was sensitive to 0.1 M 2-ME. The possible role of the reticuloendothelial system in limiting the heterogeneity of B. canis antigens in vivo is postulated.

Immunogenic characteristics of the cell culture-derived antigens were assayed by vaccination of susceptible dogs. Vaccinated dogs demonstrated a strong humoral antibody response and resisted challenge with virulent B. canis blood in that they never displayed clinical babesiosis, and no parasites were ever detected in their peripheral blood. Unvaccinated controls succumbed to babesiosis characterized by parasitemia, anemia and general lethargy. It is suggested that B. canis propagated in vitro may provide a potential source of a vaccine against canine babesiosis.

C. Study of relationship between causative agents of human sennetsu rickettsiosis and canine ehrlichiosis.

The serologic relationship between Rickettsia sennetsu, the etiologic agent of human sennetsu rickettsiosis in Western Japan, and Ehrlichia canis, the agent of canine ehrlichiosis and tropical canine pancytopenia (TCP), has been demonstrated. Using the indirect fluorescent antibody (IFA) test, the two agents cross-reacted with

convalescent canine and human sera, respectively. The degree of cross-reactivity was high, judged by homologous and heterologous titers. In the direct fluorescent antibody (FA) test, immunoglobulins from 5 patients with sennetsu rickettsiosis stained E. canis morulae contained in infected canine monocytes.

The significance of this finding was discussed in view of morphologic uniqueness of the 2 agents and a lack of their serologic relations with other major rickettsial agents.

African green monkey kidney continuous cell line (BSC-1) reported suitable for propagation of R. sennetsu was initially used to grow the organism from samples on deposit at the American Type Culture Collection (ATCC). These efforts were done two times with each of the 2 shipments of the seed material. Based upon microscopic examination of the cultures and upon the infectivity status of inoculated mice, there was no indication that we propagated R. sennetsu using the seed received from ATCC. At this stage of our efforts to establish R. sennetsu in our laboratory, we resorted to using cultures of peripheral blood monocytes derived from apparently normal human beings. The method proved useful for isolation of R. sennetsu from samples stored over a prolonged period of time.

Microscopic examination of Giemsa stained specimens of monocytes derived from the supernatant of cultures suggest that intracytoplasmic R. sennetsu underwent a specific cycle of development. Principal developmental forms of the organism in the sequence of their appearance during 20 days of incubation were loosely scattered individual

organisms, organismal clusters with individual rickettsiae easily differentiated, small and large inclusion bodies with undifferentiated individual organisms, individual organisms and inclusion bodies in large cytoplasmic vacuoles, and various cell-free organismal growth forms in close proximity to disintegrated monocytes. This development sequence appears similar to that of E. canis propagated in canine monocytes. Specific identification of R. sennetsu was made by staining cultured organisms with fluorescein-conjugated globulins extracted from pooled sera of patients convalescing from the disease. Mice infected with the cultured organism developed gross-pathologic changes indicative of infection and the organism was demonstrated in their spleens, peritoneal macrophages and mononuclear blood cells.

We introduced peripheral blood monocyte cell culture for the first successful in vitro propagation of E. canis. Since then, the method has been found useful for isolation and propagation of Neorickettsia helminthoeca, Rickettsia rickettsii, and Rickettsia tsutsugamushi. Based upon this experience and the data presented above, it appears that the peripheral blood monocyte cell cultures may be useful for an early diagnosis of sennetsu rickettsiosis by isolation of the organism from the blood of affected individuals.

**ADDENDUM:** Serodiagnostic services in support of control of blood diseases of military dogs.

In addition to conducting research under the 3 objectives as described above, this laboratory has continued with serodiagnostic services in support of the U.S. Armed Forces (Army and Air Force)

aimed at the control of hemotropic diseases (ehrlichiosis and babesiosis) among military dogs. COL V.C. Edward H. Stephenson of the Walter Reed Army Institute of Research served as a referral officer for the program. During the past year of support, a total of 1231 sera were received and 3,710 tests performed. Upon instructions from Dr. Howard E. Noyes, Director, Office and Research Management of the WRAIR, a separate contract proposal entitled, "Serodiagnostic Services in Support of Field Operation of the U.S. Armed Forces and its Canine Corps" was submitted. Approval of this contract would enable more efficient utilization of funds toward proposed research.

## II. DETAILED PROGRESS REPORT

### A. Research Accomplished Under Objective 1

Study of the effect of low-level tetracycline on infections with Ehrlichia canis for the purpose of formulating a standard operational procedure (SOP) for control of ehrlichiosis (tropical canine pancytopenia - TCP) in military dogs in endemic areas.

1. Background information: Original studies at the Walter Reed Army Institute of Research (Amyx et al., 1971) and subsequent investigations under field conditions in Thailand (Davidson et al., 1975) showed that dogs given tetracycline hydrochloride orally at a daily rate of 3 mg/l lb body weight were refractory to infection with E. canis. The indirect fluorescent antibody (IFA) test developed in this laboratory (Ristic et al., 1972) was found to be an excellent auxiliary tool to monitor the progress of control measures by detecting and quantitating anti-E. canis antibodies in dogs under study. While the above studies have demonstrated that TCP can be controlled in military dogs by daily administration of tetracycline, they have prompted a number of questions which must be answered before standard chemotherapeutic procedures for prevention of this disease can be established.

---

Amyx, H. L., Huxsoll, D. L., Zeiler, D. C. and Hildebrandt, P. K.: Therapeutic and Prophylactic Value of Tetracycline in Dogs Infected with the Agent of Tropical Canine Pancytopenia. JAVMA, 159 (1971): 1428-1432.

Davidson, D. E., Jr., Dill, G. S., Tingpalapong, M., Premabutra, S., La-Or Nguen, P., Stephenson, E. H. and Ristic, M.: Canine Ehrlichiosis (Tropical Canine Pancytopenia) in Thailand. Southeast Asian J. Trop. Med. Pub. Hlth., 6 (1975): 540-543.

Ristic, M., Huxsoll, D. L., Weisiger, R. M., Hildebrandt, P. K. and Nyindo, M.B.A.: Serologic Diagnosis of Tropical Canine Pancytopenia by Indirect Immunofluorescence. Infect. Immun., 6 (1972): 226-231.

The United States Army Medical Research unit in Kuala Lumpur, Malaysia, in collaboration with this laboratory, has undertaken a series of studies in an effort to obtain knowledge needed for formulating standard operational procedures for control of TCP. The first studies to this effect concerned application of low-level tetracycline (3 mg/lb/day) during the early phases of infection. The drug was administered 3, 7, and 14 days after infection and continued for 30 days. Results based upon actual isolation of the organism from treated dogs and serologic evidence using the IFA test showed that (1) 30 days of low-level tetracycline cleared all dogs of infection regardless of the time tetracycline was initiated, (2) all dogs treated and cleared of the infection were fully susceptible to infection and disease following reinfection, indicating that no immunity was developed and (3) transitory but relatively strong antibody responses were noted in all dogs regardless of when tetracycline therapy was instituted. (Please see Report Number VIII, 1977).

2. Examination of the effect of low-level tetracycline on dogs chronically infected with *E. canis*: Studies of the last year concerned examination of the effect of low-level tetracycline on dogs chronically infected with *E. canis*. Dogs infected for at least 60 days are considered to be chronic carriers. In these animals which have survived the acute phase of the disease, clinical signs of the disease are usually less apparent although they remain an active source of infection for susceptible dogs. Administration (30 days) of low-level tetracycline in these animals was aimed at answering the following questions: (1) Would

such treatment eliminate infections from carrier dogs, (2) what would be the effect of treatment on antibody level, and (3) would carriers freed from infection by treatment be immune to reinfection.

The experimental design used to study the effect of low-level tetracycline in chronically infected dogs is given in Table 1. On relative date 0 the following dogs were inoculated with blood from an E. canis positive donor: Nos. 149, 150, 151, 163, 164, 165, and 167. Serving as uninoculated controls were dogs Nos. 169, 171, and 172. These animals represent a primary experimental group 1 (Table 2). From relative day 60 to relative day 90, all dogs in group 1, including the controls, were dosed with tetracycline at 3 mg/lb/day per os. On relative day 122 all dogs of group 1 (including controls) were back-challenged with blood from an E. canis positive dog.

In order to establish infectious stages of dogs in group 1, blood from each of these animals was subinoculated into susceptible recipient dogs. Such inoculation sequences were as follows: (1) on relative day 74, which is 14 days after the start of tetracycline therapy, blood from group 1 was subinoculated into susceptible dogs of group 2 (Table 3); (2) on relative day 122, or 32 days after tetracycline treatment was discontinued, blood from group 1 was subinoculated into susceptible dogs of group 3 (Table 4), and (3) on relative day 186, or 64 days after back-challenge of group 1, blood from these animals was subinoculated into susceptible dogs of group 4 (Table 5).

10  
DESIGN.

Treatment and sample collections from 7 infected and 3 control dogs

---

-10 -3 0 4 11 18 25 32 39 46 53 60 67 74\* 81 88 90 95 103 110 117 122\* 124 131 137 144 151 158 -- 186\*

Infected with  
Blood of E. canis  
positive donor

Tetracycline  
therapy

Back challenge  
with blood from  
E. canis positive  
donor

\*Days on which blood from infected dogs was subinoculated into 7 susceptible dogs

Table 2.

= GROUP 1 : DOGS INOCULATED WITH BLOOD FROM A POSITIVE E. CANIS DONOR AND TREATED WITH

TETRACYCLINE BETWEEN DAYS 60 AND 90. BACKCHALLENGED ON DAY 122.

	DOG NO.									
DAY	149	150	151	163	164	165	167	169*	171*	172*
-3 (1/25/78)	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG
53 (3/22/78)	1:2560	1:2560	1:2560	1:2560	1:2560	1:2560	1:2560	NEG	NEG	NEG
88 (4/26/78)	1:320	1:640	1:640	1:320	1:1280	1:640	1:640	NEG	NEG	NEG
117 (5/25/78)	1:160	1:80	1:80	1:160	1:320	1:80	1:40	NEG	NEG	NEG
158 (7/5/78)	1:1280	1:1280	1:640	1:1280	1:640	1:1280	1:640	1:640	1:640	1:640

\* CONTROL DOGS

Table 3.

## GROUP II. RESULTS OF SUBINOCULATION 14 DAYS AFTER START OF TETRACYCLINE THERAPY.

DAY	DOG No.						
	154	155	156	161	162	166	168
0 (4/12/78)	NEG	NEG	NEG	NEG	±*	NEG	NEG
29 (5/11/78)	Pos	Pos	Pos	Pos	Pos	NEG	NEG

\*QUESTIONABLE FLUORESCENCE AT 1:10 DILUTION.

Table 4.  
GROUP III. RESULTS OF SUBINOCULATION AT 32 DAYS AFTER TETRA-  
CYCLINE TREATMENT WAS DISCONTINUED.

DAY	DOG. No.						
	142	160	170	173	176	178	180
-5 (5/25/78)	NEG	NEG	NEG	NEG	NEG	NEG	NEG
28 (6/28/78)	NEG	NEG	NEG	NEG	NEG	NEG	NEG

Table 5.  
GROUP IV. RESULTS OF SUBINOCULATION 64 DAYS AFTER BACK CHALLENGE OF GROUP I.

	Dog No.									
DAY	154*	155*	156*	161*	162*	175	179	182	183	184
-7 (7/26/78)	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG
21 (8/23/78)	Pos	No	Pos	Pos	Pos	Pos	NEG	Pos	NEG	++

\*DOGS USED IN GROUP II

\*\*NOT CLEAR POSITIVE AT 1:10.

All dogs of group 1 developed an acute infection with E. canis as demonstrated by clinical and hematologic evidence. They also developed strong antibody response in the IFA test. On day 53 post infection, all inoculated animals had strong (3+) reactions in the IFA test at a titer of 1:2560. A gradual decrease of the antibody titer, however, started at the termination of treatment and continued to do so thereafter (Table 2). Subinoculation of blood from animals of group 1 at 14 days after the start of tetracycline therapy into 7 susceptible dogs produced positive response in 5 of the recipients (Table 3). The recipient animals reverted to a negative serologic status at 75 days after inoculation, indicating that the infectious dose was not sufficient to establish durable (chronic) infection in the recipient animals. Results of subinoculation at 32 days after tetracycline treatment are given in Table 4. None of the 7 recipient animals contracted infection, suggesting that at this stage, all treated animals of group 1 were free of E. canis. Finally, Table 5 gives results of serologic responses of susceptible animals subinoculated with the blood of animals from group 1 at 64 days after back-challenge of these animals. As indicated by serologic response, 4 of the recipient animals contracted infection, no infection occurred in 2 dogs, and the infection of the remaining dog was questionable.

Since on back-challenge the animals of group 1 showed little clinico-hematologic response in comparison with the response on primary infection, it is indicated that these animals possessed a degree of protective immunity. Based upon data of Table 5,

this immunity was sufficient to reject the infection in some (at least 2) but not all (the remaining 4) animals.

3. Summary: Administration of low-level tetracycline 3 mg/lb/day per os was shown to be effective in freeing dogs from the chronic (carrier) E. canis infection. A slow but continuous decline in antibody titer using the indirect fluorescent antibody (IFA) test was indicative of the efficacy of the drug to destroy residual infection in these dogs. As evidenced by back-challenge, chronically infected dogs freed from E. canis by tetracycline possessed a considerable degree of protective immunity. This immunity seems to be more efficient in protecting the animal from development of clinical disease rather than reinfection.

B. Research Accomplished Under Objective 2:

Development of a method for in vitro cultivation of Babesia canis and use of culture-derived antigen as immunogen for canine babesiosis.

1. Background information: The significance of canine ehrlichiosis and babesiosis as an operational problem in military dogs has been explained and documented in previous reports. Although there is a need for additional studies to further elaborate on the use of tetracycline as a preventive and/or therapeutic method under various epidemiologic conditions as indicated in the results under Objective 1, it is evident that an effective control method for canine ehrlichiosis is at hand. The development of an immunoprophylactic method for canine ehrlichiosis would be advantageous to the tetracycline method, however, based upon

current understanding of protective immunity of this disease, prospects for such a vaccine are not favorable. The situation with canine babesiosis from the standpoint of its control by chemotherapy seems opposite to that of ehrlichiosis. There are no effective therapeutic and chemoprophylactic methods for canine babesiosis. Studies of the mechanism of protective immunity with various Babesia species, however, showed that protection can be induced by inactivated immunogens.

The aim of this study was to develop a cell culture method for production of Babesia canis antigens which may be used as immunogens against canine babesiosis.

2. Material and methods:

a. Source of Babesia canis used for cultures and challenge.

Blood from dog 309 (chronic B. canis carrier) was collected in 20% ACD solution and injected into a susceptible splenectomized dog (BG-1). The blood of this dog was used to initiate cell cultures. One additional passage into another splenectomized dog (BG-2) was made in order to increase the virulence of the strain. Infected blood from the latter dog was used to challenge the experimental animals. Each challenged animal received  $10^8$  Babesia parasites administered intravenously.

b. Medium. Preliminary studies indicated that modified Roswell Park Memorial Institute (RPMI) 1640 medium was ideal for primary cultivation of B. canis. The medium was prepared as described by Trager and Jensen (1977) for the cultivation of

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Trager, W. and Jensen, J.: Plasmodium falciparum in Culture: Use of Out-dated Erythrocytes and Description of the Candle Jar Method. J. Parasitol., 63 (1977): 883-886.

Plasmodium falciparum. Briefly, the modified RPMI 1640 contained N-2-hydroxyethylpiperazine, N'-2-ethane sulfonic acid (HEPES) at a final concentration of 25 nM with a pH of 6.75. Nine hundred ml of this solution were adjusted to 960 ml with double distilled water and sterilized using a 0.22  $\mu$  millipore filter. The medium was dispensed aseptically into 100 ml aliquots and stored at 4° C for a predetermined maximum period of 1 month. Before use, the pH of the medium was adjusted to 7.4 by addition of 4.2 ml of a sterile 5% solution of sodium bicarbonate (NaHCO<sub>3</sub>) to each 100 ml aliquot of medium. Normal dog serum was added to a final concentration of 20%. No dog serum was added to the medium for washing and storage of infected erythrocytes. The latter medium was designated washing and storage medium (WSM).

- c. Collection of blood and preparation of primary cell culture. When the inoculated dogs attained a parasitemia of 3 - 4%, they were aseptically exsanguinated under general anesthesia. Blood was collected in 500 ml bottles containing glass beads for defibrination. The defibrinated blood was centrifuged at 400 g for 20 minutes at 4° C. The supernatant and buffy coat were aseptically removed and the serum saved for future studies. The erythrocytes were washed 3 times in WSM at 400 g for 20 minutes at 4° C. Some of the washed erythrocytes were resuspended and stored in this medium at 4° C until required for culture. The rest of the cells were divided into 12 ml aliquots and maintained in 50 ml spinner flasks.<sup>a</sup>

---

<sup>a</sup>Bellco Glass Inc., Vineland, NJ.

To one volume of washed cells, 4 volumes of culture medium were added. The spinner flasks were incubated at 38° C in a humidified atmosphere of 5% CO<sub>2</sub>. The cells were maintained in uniform suspension by gentle magnetic stirring. After 48 hours, the cultures were centrifuged at 400 g for 10 minutes at 4° C. The supernatant was saved; fresh culture medium was added and incubation continued for a further 48 hours (i.e., a total of 96 hours of cultivation). The 96-hour cultures were centrifuged at 400 g for 10 minutes at 4° C. The supernatant was saved and the pellet, composed predominantly of uninfected erythrocytes was discarded. The cultures were discontinued at this point. New cultures were prepared in the same way using infected cells that had been maintained at 4° C in WSM. Giemsa-stained smears were made every 24 hours from each spinner flask. Five hundred erythrocytes were counted per slide to determine the percent infection. The number of extracellular (E) and intracellular (I) forms of the organism was recorded. The hematocrit from each culture was also determined to monitor the stability of the erythrocytes. For evaluation of the kinetic relationship between E and I, the ratio E/I was computed and plotted against duration time in culture. The total number of extracellular forms ( $E_t$ ) per flask was determined every 24 hours by the following formula:

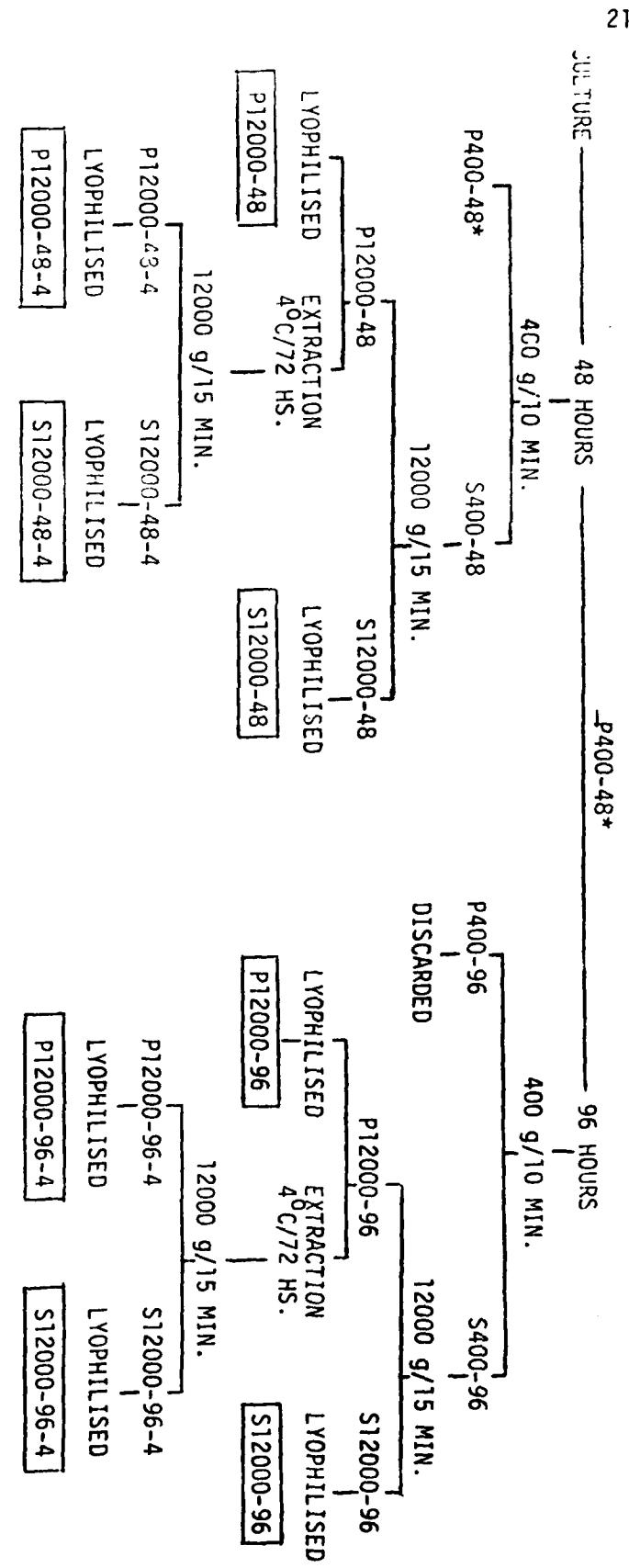
$$E_t = E_{500} \times \frac{N}{500}$$

where  $E_{500}$  represents the number of extracellular parasites per 500 cells and N the total erythrocyte count per flask.

For control purposes, a spinner flask containing normal canine (uninfected) erythrocytes was maintained under identical conditions to those of the B. canis infected cultures.

- d. Isolation of Babesia canis antigens. The procedure for isolation of antigens derived from B. canis primary cell cultures is summarized in Figure 1. Forty-eight hours and 96 hours post cultivation, the cultures were centrifuged at 400 x g for 10 minutes at 4° C. The pellet (P400-48) containing infected erythrocytes was resuspended in culture medium and incubation was continued as described above. The pellet (P400-96) was discarded. The supernatant (S400) was further centrifuged at 12000 x g for 15 minutes at 4° C. The supernatant (S12000) was divided into 10 ml aliquots and lyophilized. One portion of the pellet (P12000), which contained extracellular forms of B. canis, was also divided into 10 ml aliquots and lyophilized. The rest of P12000 was resuspended in PBS and subjected to extraction at 4° C for 72 hours by continuous magnetic stirring. After 72 hours, this suspension was centrifuged at 12000 x g for 15 minutes. The supernatant was removed, lyophilized and stored. One volume of the pellet was resuspended in 9 volumes of PBS, lyophilized and stored. All the fractions were tested in gel diffusion for antigenic activity. Supernatants (S12000) and pellets (P12000) were concentrated 14 times and 4 times, respectively, by adding distilled water to each 10 ml aliquot of lyophilized material prior to use in the in vitro tests.

Figure 1



e. Electron microscopy. Cultures after various periods of incubation were centrifuged at 400 x g for 10 minutes at 4° C. The supernatant was removed and centrifuged at 12000 x g for 15 minutes. The resultant pellet (P12000), containing the extracellular forms of B. canis, was resuspended in 10 volumes of fixative comprised of 2% gluteraldehyde, 0.1 M cacodylate buffer and 4% sucrose at a final pH of 7.4. The suspension was centrifuged at 200 x g for 10 minutes and the supernatant discarded. Fresh fixative was added in gross excess of sample. Such fixed samples were stored at room temperature until processed for transmission electron microscopy.

f. In vitro tests.

Indirect fluorescent antibody (IFA) test. The test (Ristic, et al., 1972) was used to monitor the immune response of dogs inoculated with soluble and corpuscular antigens derived from the B. canis primary cell culture and challenged with a homologous strain of the parasite. Blood films containing approximately 20% infected cells and smears containing extracellular forms of B. canis obtained during the cultivation of the organism were removed from the freezer (-65° C) and placed in a desiccator jar over anhydrous calcium chloride. The jar was evacuated and the slides left in the vacuum for 1 hour at room temperature. The slides were fixed in absolute acetone for 20 minutes and air dried for 15 minutes. Circled areas (1 cm in diameter) were flooded with different dilutions of test sera. Appropriate positive and negative control sera were included with each run. The slides were placed in a humidified chamber

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Ristic, M., Huxsoll, D., Weisiger, R., Hildebrandt, P., and Nyindo, M.B.A.: Serologic Diagnosis of Tropical Canine Pancytopenia by Indirect Immunofluorescence. *Infect Immun.*, 6 (1972): 226-231.

and incubated at 37° C for 30 minutes. They were rinsed twice in PBS for 5 minutes each time followed by rinsing in distilled water for 4 minutes. The slides were finally air-dried thoroughly. Fluorescein conjugated anti-canine globulin was applied. Incubation and rinse procedures were repeated as above. Mounting fluid containing 9 parts of glycerin and 1 part of PBS was placed on each slide and all antigen spots were covered with a coverslip (22 by 44 mm). The slides were examined under a microscope<sup>b</sup> equipped with an ultraviolet light source, a BG-12 exciting filter, an OG-1 barrier filter, a dark field condenser and an automatic 35 mm camera.

Gel diffusion. The technique of Ouchterlony (1962) was used with only minor modifications. The agar medium consisted of 1 gm of agarose and 100 ml of 0.15 M sodium chloride containing merthiolate at a final concentration of 1:10,000. The agar was dissolved in saline by boiling for 10 minutes in a water bath and dispensed in 4 ml quantities on 1 x 3 inch microscope slides. The slides were stored at room temperature or 4° C in a humidified chamber. Wells, 3mm in diameter and 3 mm apart were cut in the agar immediately prior to use. These wells were filled with the reagents once and tests were performed at room temperature. Precipitin lines were usually visible after 8 hours, however, the final reading was recorded after 48 hours of incubation.

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<sup>b</sup>Microscope Leitz Ortholux, E. Leitz Incorporated, NY.

Ouchterlony, O.: Diffusion-in-Gel Methods for Immunological Analysis. II. In Progress in Allergy, Vol. VI (1962) pp. 30-154, ed. Kallos, P. and Waksman, B. H., Basel and New York

Ammonium sulphate precipitation. Saturated ammonium sulphate was added to S12000 to a final concentration of 30% V/V. The effect of 50% ammonium sulphate was also investigated. The saturated ammonium sulphate was added dropwise to a magnetically agitated sample in an ice bath. The reaction was allowed to proceed for 15 minutes and the mixture was centrifuged at 12000 x g for 15 minutes. The resultant pellets and supernatants were dialyzed against several changes of 0.15 M sodium chloride solution until no sulphate ions were detectable using the barium chloride precipitation test. The presence of soluble antigen was checked by immunodiffusion against B. canis immune serum.

Test for 2-mercaptoethanol (2-ME) sensitivity. The reducing agent 2-ME<sup>c</sup> was added to the antigen solution to a final concentration of 0.1 M. The mixture was incubated at 37° C for 30 minutes. The control system comprised of isotonic saline instead of 2-ME was held at 37° C for 30 minutes. The treated samples were dialyzed against 0.15 M sodium chloride containing iodoacetamide for 24 hours. The samples were reconstituted to the original volume and tested for antigenicity relative to unreduced controls.

Test for thermostability. Antigen solution (S12000) was heated in a water-bath at 37° C for 30 minutes, 56° C for 30 minutes, 65° for 15 minutes and 100° C for 15 minutes. The samples were allowed to cool at room temperature before testing them in immunodiffusion.

Gel filtration on Sephadex G-200. The system was used to compare elution profiles of soluble antigens present in the serum of acutely infected dogs with elution profiles of cell culture-derived soluble

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<sup>c</sup>Bio-Rad Laboratory, Richmond, CA.

antigens. Sephadex G-200<sup>d</sup> was equilibrated in 0.15 M sodium chloride solution. A column (75 x 2.5 cm) was prepared and allowed to stabilize by downward elution with a peristaltic pump at a previously established rate of 16 ml/hr. The column was calibrated for 19 S, 7 S, and 5 S molecules using whole canine serum as a marker. Two ml of sample was carefully layered onto the column. Uniform loading was achieved by overlaying the sample with 10% dextrose solution. Positive downward pressure elution was adjusted to give 7 ml fractions every 20 - 30 minutes. The absorbance at 280 nm was automatically monitored and recorded by a UA-5 UV monitor recorder. Tubes corresponding to various peak-segments were pooled and lyophilized. The concentrated material was reconstituted to the original volume (2 ml) with distilled water. Gel diffusion analysis for antigen was carried out on all peaks for molecular weight estimation.

- g. Preparation and administration of *B. canis* immunogens. Five hundred microliters of antigen (S12000-48) was placed in a crucible and dried to constant weight in a 50° C oven. The same volume of uninfected S12000-48 was treated under the same conditions. The difference in weight between S12000-48 antigen and S12000-48 control was attributed to the presence of soluble antigen. Based on dry weight analysis, the antigen concentration was adjusted to give 43 mg/ml.

The number of extracellular forms of *B. canis* (corpuscular antigen) was determined as described above. Approximately 2 mg of soluble antigen per pound of body weight or  $5 \times 10^8$  extracellular forms of *B. canis* was injected into the experimental dogs. One mg

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<sup>d</sup>Pharmacia, Uppsala, Sweden.

(60 ml) of a 1.5% aqueous solution of Saponin-Quil A (Dalsgaard, 1974) was added to each dose of soluble and corpuscular antigens to test the immunopotentiation of the adjuvant. The mixture was gently agitated for 15 min and injected subcutaneously into the dogs (Table I).

### Results

1. Relationship between intracellular and extracellular forms of Babesia canis and total erythrocyte count. The relative distribution of extracellular (E) and intracellular (I) forms of B. canis at different times during culture are shown (Fig. 2-A). The ratio of extracellular to intracellular forms is plotted against duration in primary cell culture. In the same figure, changes in hematocrit values are shown.

At 0 hours in culture, the mean E/I was 0, corresponding to a percent infection averaging 3.85% and a hematocrit of 11%. After 24 hours in culture, the mean E/I was 0.75, corresponding to a percent infection averaging 7.5%. The hematocrit remained stationary. The percent infection began to decline while the E/I ratio increased as the number of extracellular forms rose. By 48 hours, the mean percent infection was 3.3% and the E/I ratio was 4.06. The hematocrit at this point was still unchanged. At 72 hours of cultivation the ratio E/I had reached 9.08 and the mean percent infection had dropped to 1.75%, and the hematocrit began to decline very sharply. There were very few intracellular forms observed at 96 hours of cultivation and the E/I ratio was 26.83 corresponding to a percent infection of 0.6%, and the hematocrit had dropped to 4.2% average.

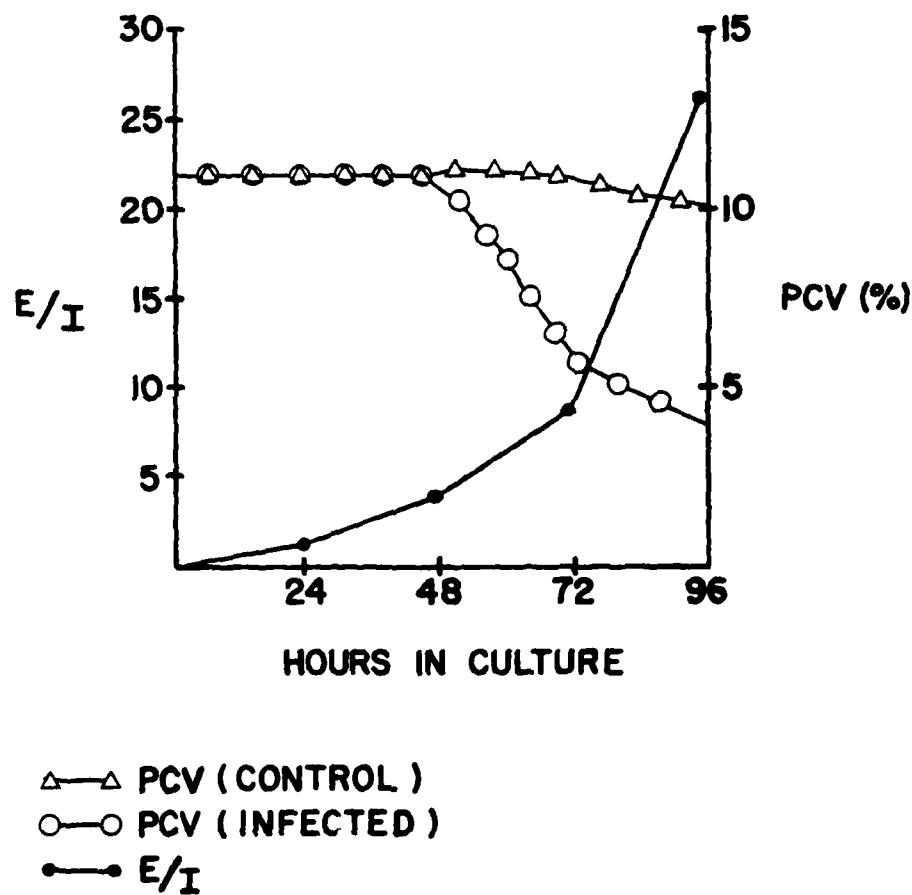
TABLE I. DISTRIBUTION OF DOGS USED IN TESTING IMMUNOGENICITY OF CELL CULTURE-DERIVED BABESIA CANIS ANTIGENS

GROUP	NO. OF DOGS	IMMUNOGEN	ADJUVANT	CHALLENGE
1	3	a-soluble ag	Saponin (Quil-A)	
		b-soluble ag	Saponin (Quil-A)	<u>B. canis*</u>
		c-soluble ag	-----	
2	3	a-corpuscular ag	Saponin (Quil-A)	
		b-corpuscular ag	Saponin (Quil-A)	<u>B. canis*</u>
		c-corpuscular ag	-----	
3	3	a- -----	Saponin (Quil-A)	
		b-normal canine erythrocyte culture	Saponin (Quil-A)	<u>B. canis*</u>
		c-canine erythrocyte stroma	-----	

\*Blood from dog BG-2 obtained during the acute phase of infection and containing  $10^8$  parasite was used to challenge animals in this experiment.

FIGURE 2-A

RELATIONSHIP BETWEEN EXTRACELLULAR (E) AND INTACELLULAR (I) FORMS OF B. CANIS AND PACKED CELL VOLUME (PCV). RESULTS REPRESENT MEAN OF 8 SPINNER FLASKS.



The hematocrit in the control culture underwent only slight changes during the last 24 hours showing a shift from a value of 11% to 10%. Detailed results of the values obtained from 8 spinner flasks and one control culture are shown in Table II. Absolute counts of the extracellular and intracellular parasites were monitored in relation to changes in hematocrit, total erythrocyte counts and percent infection. The highest number of total extracellular ( $E_t$ ) was observed at 48 hours of cultivation.

2. Morphological and ultrastructural studies on different stages of Babesia canis derived from primary cell culture.

Light microscopy. The different stages of development of B. canis in primary cell culture are illustrated in Figures 2 - 7.

In Figure 2, the smallest extracellular and intracellular forms of B. canis are shown. They are approximately  $1 \mu$  in diameter and round shaped. Multiple infection of the reticulocyte-like cells is also a prominent feature. In general, at least 4 organisms were found in each of these cells. The cell in the center of Figure 2 contained 16 parasites, the highest number recorded. This degree of multiple infection was never observed in smears made directly from blood of an infected dog. In Figure 3, some extracellular forms of the parasite are seen attaching to an erythrocyte, presumably at the initial stage of invasion. Forms of the parasite in a "budding" stage are shown in Figs. 4 and 5. Multiple intracellular forms of the organism in a characteristic ring arrangement are shown (Fig. 6). A similar pattern of the organism was observed extracellularly (Fig. 7). Such forms were never observed in smears prepared directly from

TABLE II. CHANGES IN HEMATOCRIT (HCT), TOTAL RED BLOOD CELLS COUNT, INTRACYTOPLASMIC FORMS (I), EXTRACELLULAR FORMS (E), RATIO OF EXTRACELLULAR (E) TO INTRACELLULAR (I) FORMS, TOTAL NUMBER OF EXTRACELLULAR FORMS ( $E_t$ ) AND PERCENT INFECTION IN BABESIA CANIS PRIMARY CELL CULTURES\*.

HOURS	HCT. (%)	BLOOD CELLS ( $10^{10}$ )	I	E	E/I	$E_t$ ( $10^9$ )	% INFECTION
0	11	7.5	19.25	0	0	0	3.85
24	11	7.5	37.75	28.37	0.75	4.3	7.55
48	11	7.5	16.62	67.50	4.06	10.1	3.32
72	5.75	3.9	8.87	80.62	8.28	6.33	1.77
96	4	2.8	3.0	80.50	26.83	4.47	0.60
C.C:**0-96	10.80	7.8	0	0	0	0	0

\* Mean values from eight spinner flasks.

\*\* Control Culture

% Infection, I and E were determined by counting 500 cells per blood smear.  
Extracellular (E) forms were not included in % infection.



Figure 2. Giemsa-stained smear showing the smallest infective units of Babesia canis. Note also (arrow) 16 trophozoites in one cell X 850.

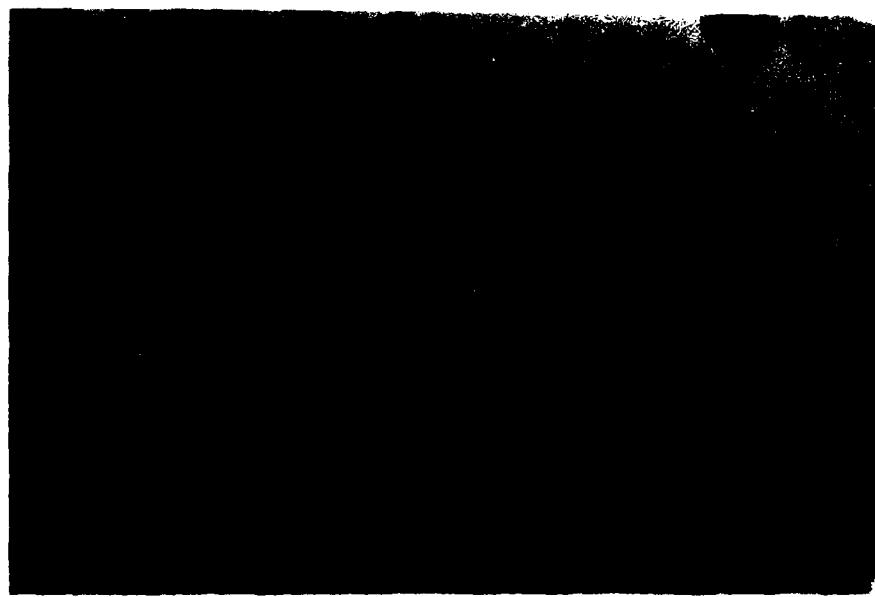


Figure 3. Giemsa-stained smears showing extracellular forms of Babesia canis (E) attaching to the erythrocyte. Single intracellular forms (S) and even-numbered trophozoites (T) can also be seen X 850.



Figure 4. Multiple "budding" forms of dividing Babesia canis (B) (arrow). Giemsa staining method X 850.



Figure 5. A single "bud" (arrow) originating from the mother cell, leading to the formation of two piriform parasites. Giemsa staining method X 850.



Figure 6. The typical ring-forms (R) of Babesia canis in culture. Note also some extracellular forms of the organism (E). Giemsa staining method X 850.

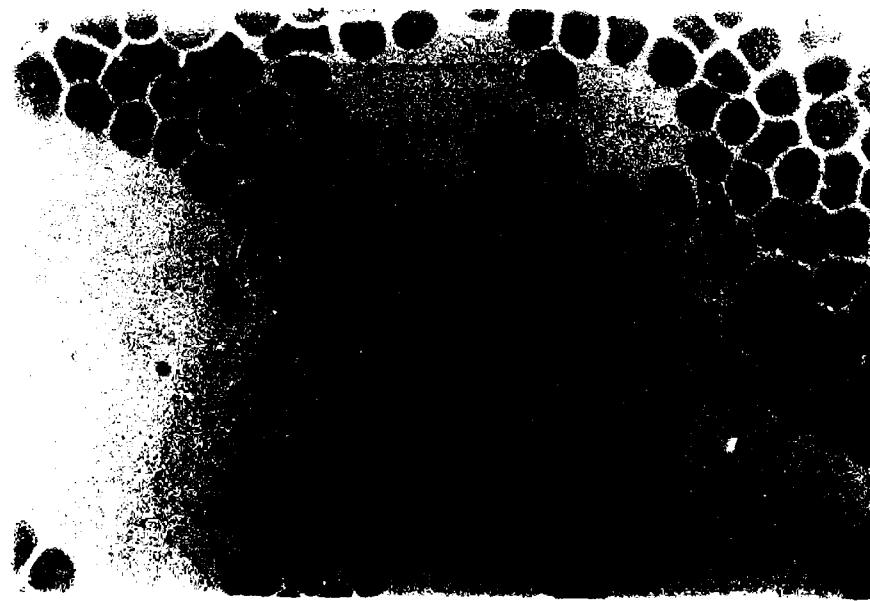


Figure 7. An extracellular chain of Babesia canis organisms reminiscent of the ring-forms previously observed intracellularly (arrow). Giemsa staining method X 850.



Figure 8. Numerous extracellular forms in the P12000 pellet from the supernatant of a 96-hour culture of Babesia canis. This pellet contained a high concentration of corpuscular Babesia canis antigen. Giemsa staining method X 850.

blood of an infected dog. As shown in Figure 8, the pellet (P12000) contained massive numbers of extracellular forms. This pellet was subjected to electron microscopy.

Electron microscopy. The organism observed in this study had a characteristic piriform (Figs. 9, 10, 11, 12), and round shape (Fig. 13). They were limited by a clearly defined double membrane. A third membrane, probably host-derived, was also observed. A small mass of vacuolated cytoplasm attached to the posterior end (Figs. 9 and 12) appears to be the only remnant of the original mother cell after the "budding" process, leading to the formation of the daughter cells. Anterior polar rings were present in the organisms and appeared as thickening of the inner membrane (Figs. 9, 12). Rhoptries and micronemes were concentrated in the anterior end (Figs. 11, 12). A cytostome was seen in the pellicle of one parasite (Fig. 10). Nuclear pores were observed in the double membrane which delimits the nucleus (Fig. 11). Mitochondria were identified and appeared as double membrane organelles (Figs. 9, 11). Intracytoplasmic electron dense particles considered to be food vacuoles were observed occasionally (Figs. 9, 12). The cytoplasm contained an extensively developed network of endoplasmic reticulum (Figs. 9, 10). The outer membrane of the free B. canis merozoites was covered with a distinct surface coat (Fig. 13).

3. Isolation and partial characterization of Babesia canis antigens derived from primary cell culture. The isolation procedure of the soluble and corpuscular antigens by differential centrifugation and extraction at 4° C is presented in Figure 1. The soluble antigen



Figure 9. Electron micrograph of two new "budding" Babesia canis parasites within an erythrocyte. The organism shows rhoptries (Rh), micronemes (Mn), polar rings (PR), double cytoplasmic membrane (DM), a probable host origin membrane (HOM), food vacuoles,(FV), mitochondria (Mi), endoplasmic reticulum (ER), remnants of mother cell (V), nucleus (N) and an undifferentiated daughter cell (UDC) X 20,000.



Figure 10. Electron micrograph of a merozoite of *Babesia canis*. A cytostome (C), double membrane (DM), endoplasmic reticulum and the probably host-origin membrane are observed X 90,000.

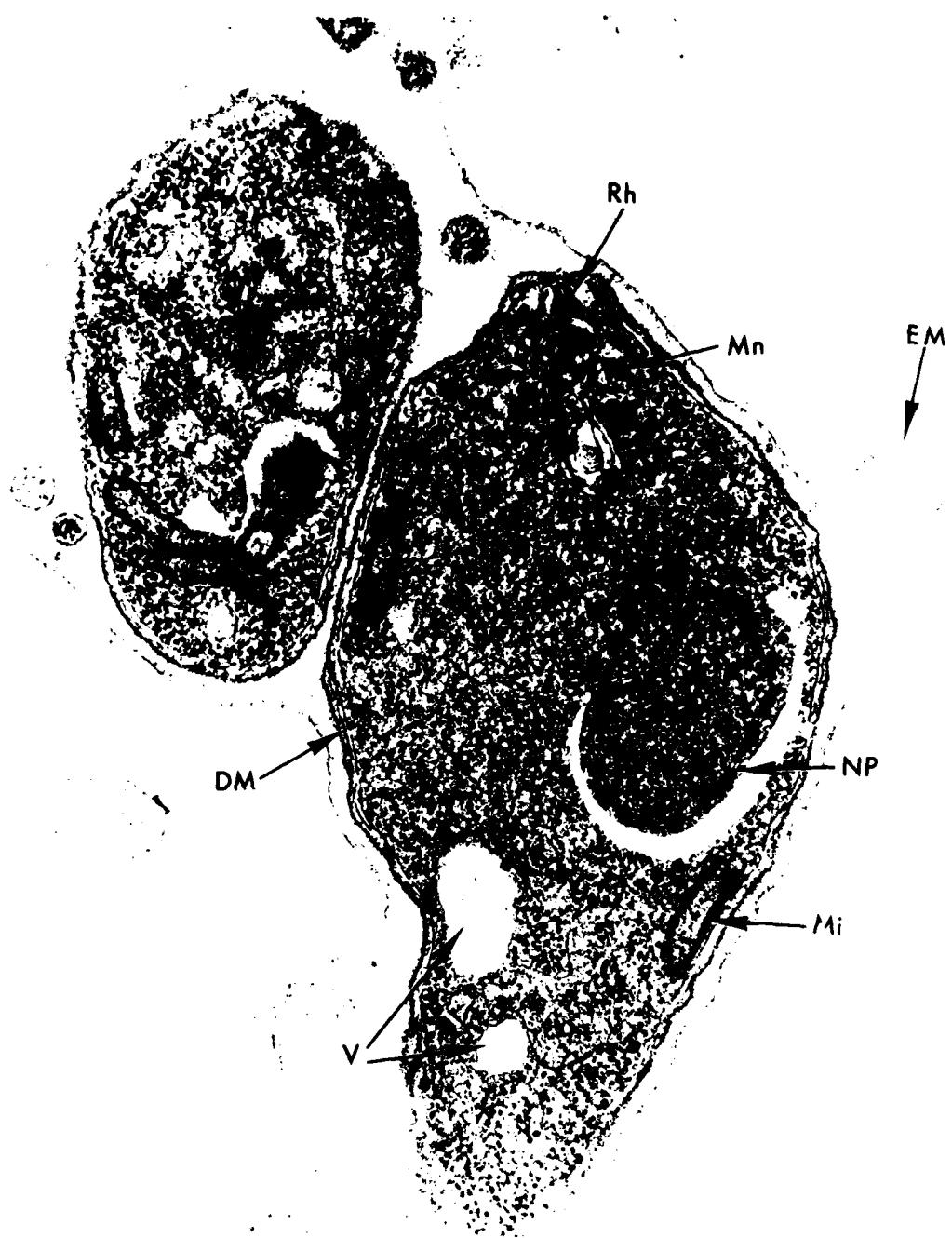


Figure 11. Electron micrograph of the merozoites surrounded by an erythrocytic membrane (EM). Note rhoptries (Rh), micronemes (Mn), nucleus (N), nuclear pores (NP), mitochondria (Mi), vacuoles (V) and double membrane delimiting the parasite X 50,000.



Figure 12. Electron micrograph of a merozoite within an erythrocyte. Polar rings (PR), rhoptries (Rh), food vacuole (FV) and vacuolated remnants of mother cell (V) are shown X 52,000.

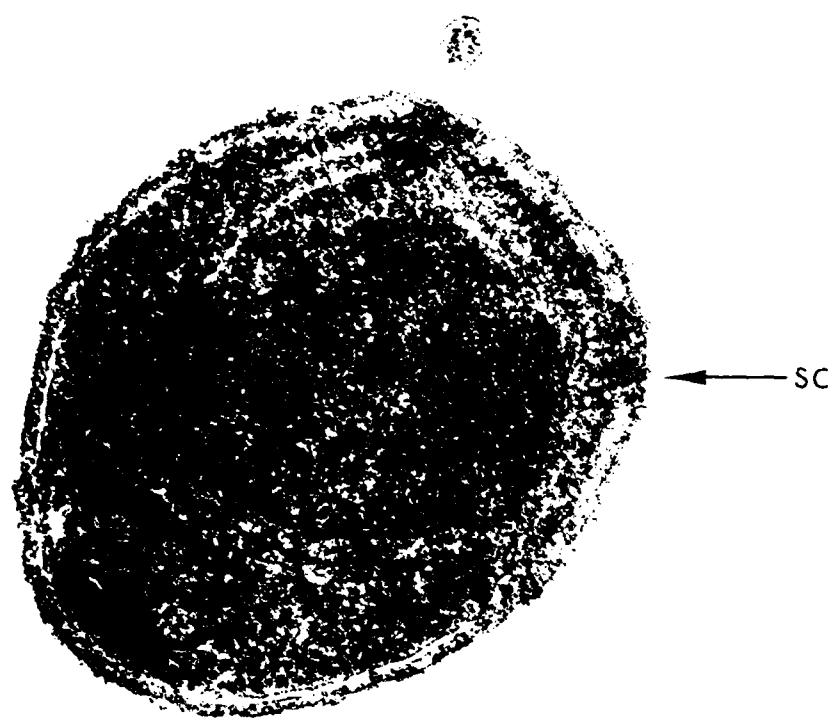


Figure 13. A free Babesia canis merozoite. Note the prominent nucleus (N) and the very distinct surface coat (SC) X 45,000.

(S12000) was characterized by various methods and the results are described below.

Immunodiffusion. The immunodiffusion results are shown (Fig. 14). When tested against anti-B. canis sera obtained from a carrier dog (309), the S12000 fractions, 48 and 96 hours post cultivation, gave 4 - 5 precipitin lines. Prior to extraction of the pellet (P12000) at 4° C, the resultant preparation (P12000-48 and P12000-96) also gave 4 - 5 precipitin lines against the same antiserum. After extraction at 4° C, the pellet (P12000-48-4 and P12000-96-4) gave weaker precipitins showing 3 distinct lines. The corresponding supernatants before and after extraction at 4° C gave similar patterns of reactivity as demonstrated for the precipitates. Serum collected from a dog suffering from acute babesiosis gave 3 precipitin lines against homologous immune serum (309). A number of antigenic determinants were common to S12000, P12000 and acute serum antigens.

Precipitation with Ammonium Sulphate. The soluble antigenic components of S12000 were not precipitated with 30% ammonium sulphate. By raising the concentration of ammonium sulphate to 50%, most of the antigenic moiety was precipitated, leaving only limited reactivity in the supernatant (Fig. 15).

Tests of Stability of Babesia canis Soluble Antigens to 2-Mercaptoethanol (2-ME) and Heat. Treatment of the S12000 antigens with 0.1 M 2-ME at 37° C for 30 minutes abolished all the antigenicity. The antigen activity was stable at 37° C for 30 minutes, 56° C for 30 minutes and 65° C for 15 minutes, but was completely inactivated by heating at 100° C for 15 minutes (Table 3).

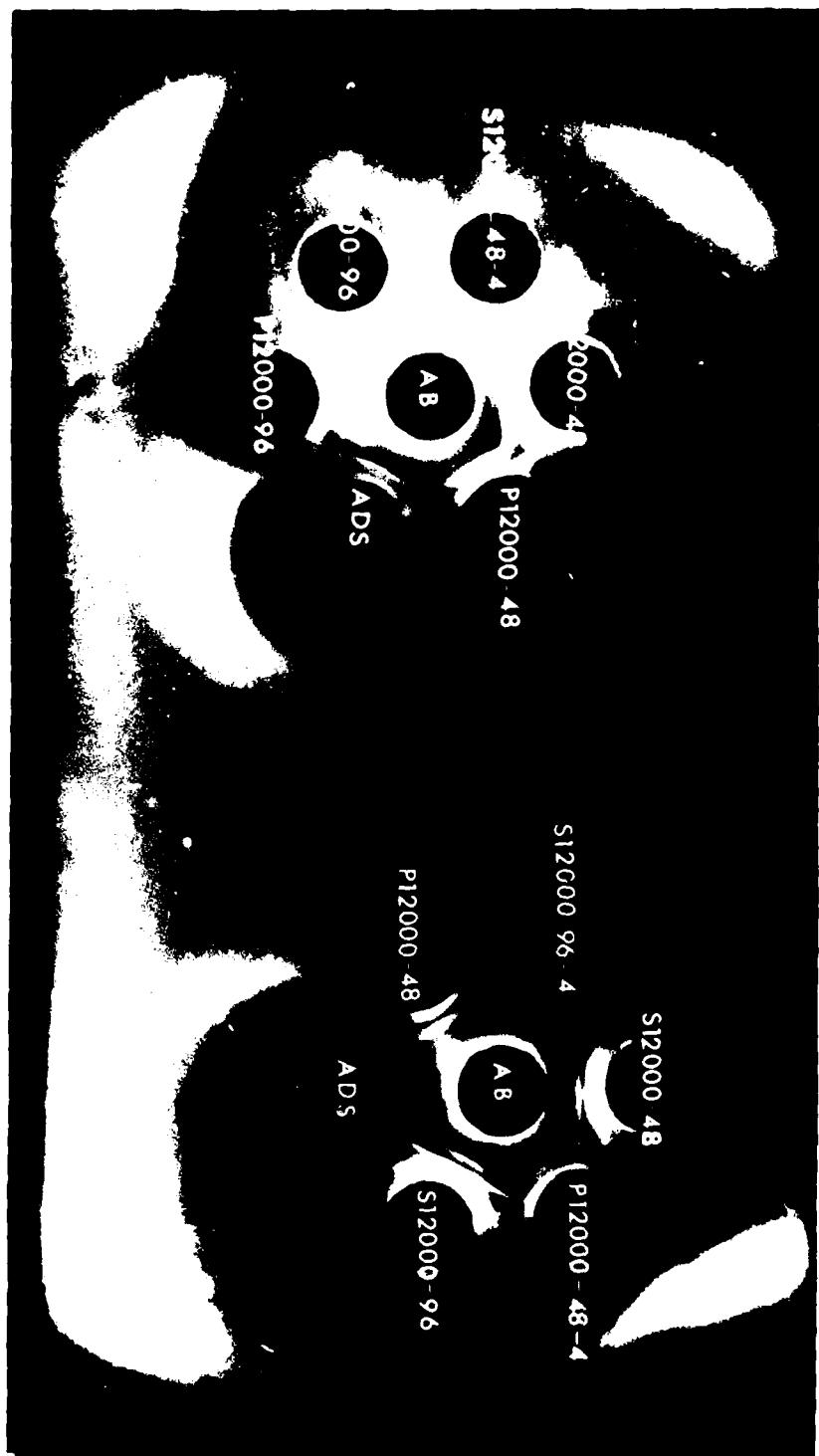
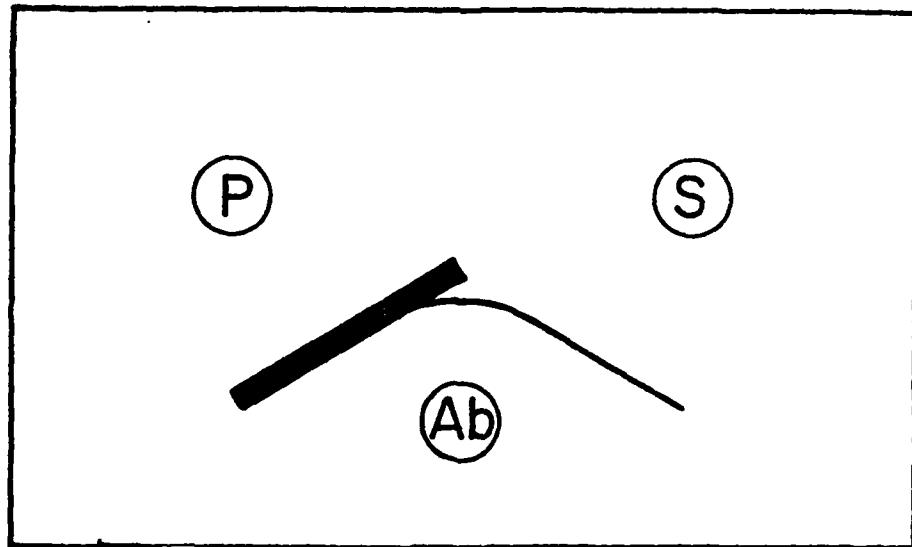


Figure 14. Immunodiffusion in gel of *Babesia canis* antigens derived from primary cell culture. These antigens were compared with those obtained from acute serum.

- | Left:           | Right:          |
|-----------------|-----------------|
| 12. S12000-48   | 12. S12000-48   |
| 2. P12000-48    | 2. P12000-48-4  |
| 4. Acute serum  | 4. S12000-96    |
| 6. P12000-96    | 6. Acute serum  |
| 8. S12000-96    | 8. P12000-48    |
| 10. S12000-48-4 | 10. S12000-96-4 |

AB = Immune serum from a *B. canis* recovered dog.

Figure 15



Schematic representation of the immunodiffusion reactions of S12000 following treatment with 50% Ammonium Sulphate.  
NOTE: most of the antigenic determinants were found in the precipitate (P) and only limited reaction was detected in the supernatant (S). Treatment with 30% Ammonium Sulphate left all the antigenic activity in the supernatant. The fractions were tested against anti-Babesia canis serum (AB).

TABLE 3. CHEMICAL AND HEAT STABILITY OF *B. CANIS* SOLUBLE ANTIGEN  
(S12000)

TREATMENT	TIME OF EXPOSURE	RESULTS*
0.1 M 2-ME	30 min.	-
100°C	15 min.	-
65°C	15 min.	+
56°C	30 min.	+
37°C	30 min.	+

\* Tested against immune serum by gel diffusion.

Molecular Sieve Chromatography on Sephadex G-200. When S12000 was fractionated on a G-200 column, all the antigenic activity was co-eluted with IgM in the molecular range of 900,000. Antigen associated with acute serum was detected exclusively in the region of albumin and hemoglobin, corresponding to a molecular weight ranging between 67,000 and 70,000 (Fig. 16).

4. Immunogenic studies on soluble and corpuscular antigens of Babesia canis derived from primary cell culture.

Humoral antibody response. Antibody responses in 3 dogs of group 1 inoculated with cell culture-derived soluble B. canis antigen in combination with Saponin (Quil A) adjuvant (2 dogs) and those of group 2 (3 dogs) which received corpuscular (merozoite) antigens plus the adjuvant (2 dogs) are given as an example in Figures 17 (dog 276) and 18 (dog DDC03), respectively. A maximum primary antibody response in dog 276, which received soluble antigen + adjuvant, occurred on day 8 following inoculation while such a response in dog DDC03, which received corpuscular antigen + adjuvant, was evident on day 18 post vaccination. The second vaccine (booster) dose was given 30 days after the first dose. Dogs of both vaccine groups responded rapidly after a booster dose and reached a maximum titer of 1:2560 8 days after the injection. Thereafter, antibody titers of dogs in both experiments declined to levels of 1:640 and 1:1280. After the challenge which was administered on day 55 following the first vaccine dose, dogs of both vaccine groups showed a rapid antibody rise reaching a level of 1:20480 during 20 to 30 days after challenge.

Soluble and corpuscular antigens administered without adjuvant induced antibody responses of 1:320 - 1:640 only after administration

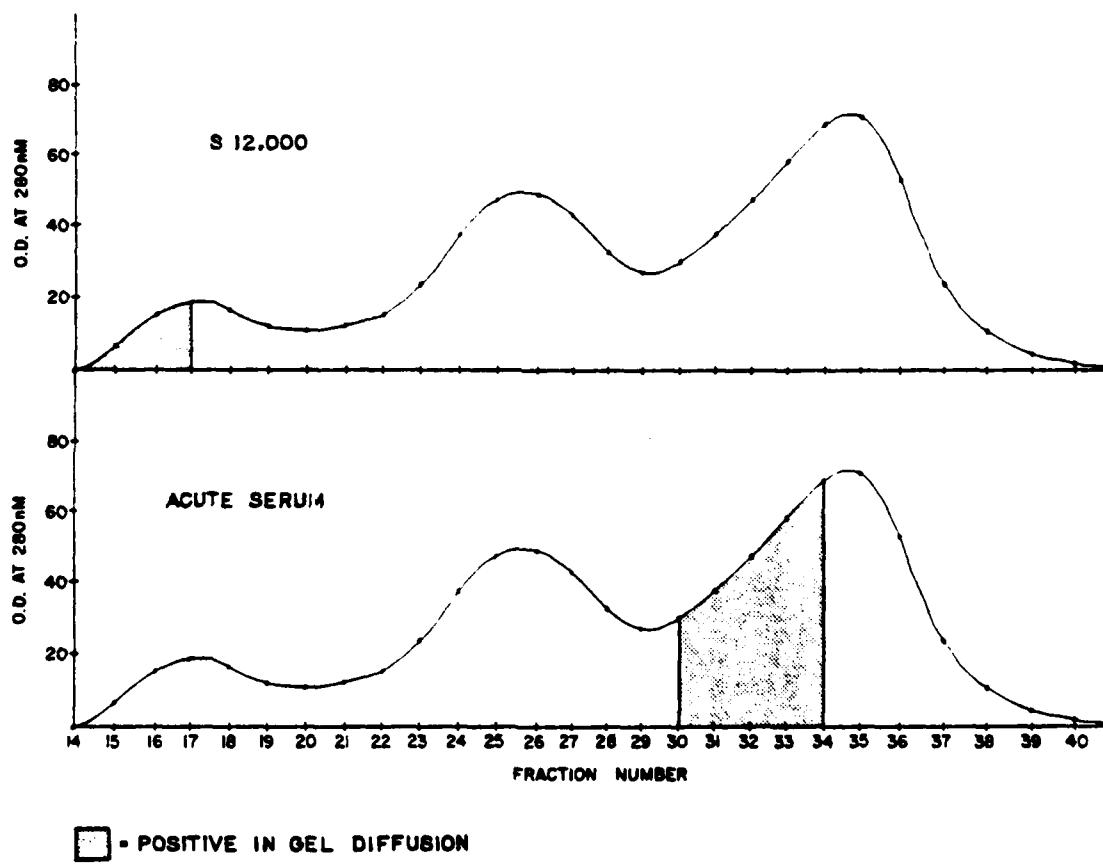


Figure 16. Elution profile of S12000 and acute serum on a Sephadex G-200 column calibrated with normal dog serum. Shaded areas show position where antigen was detected by immuno-diffusion against serum from a dog recovered from babesiosis. NOTE: Only large molecular weight antigen was detected in S12000 whereas only small molecular weight moieties were demonstrated in acute serum.

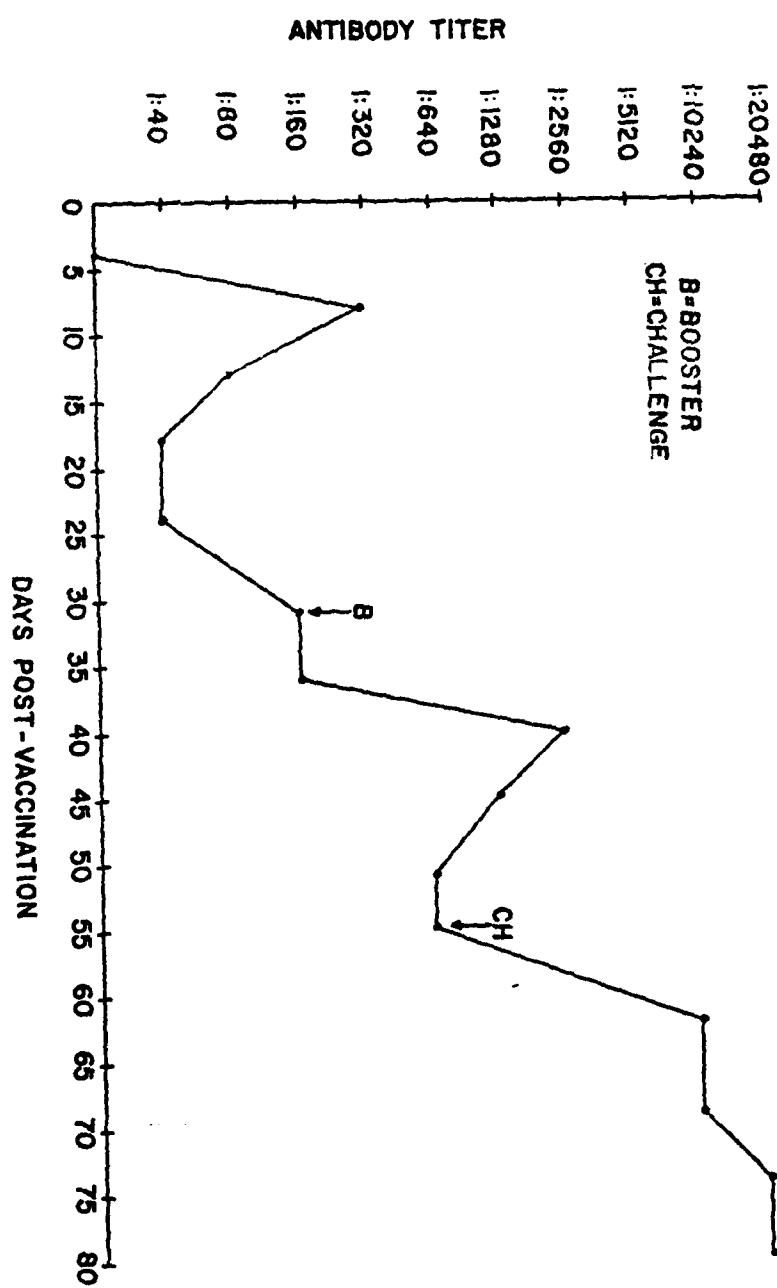
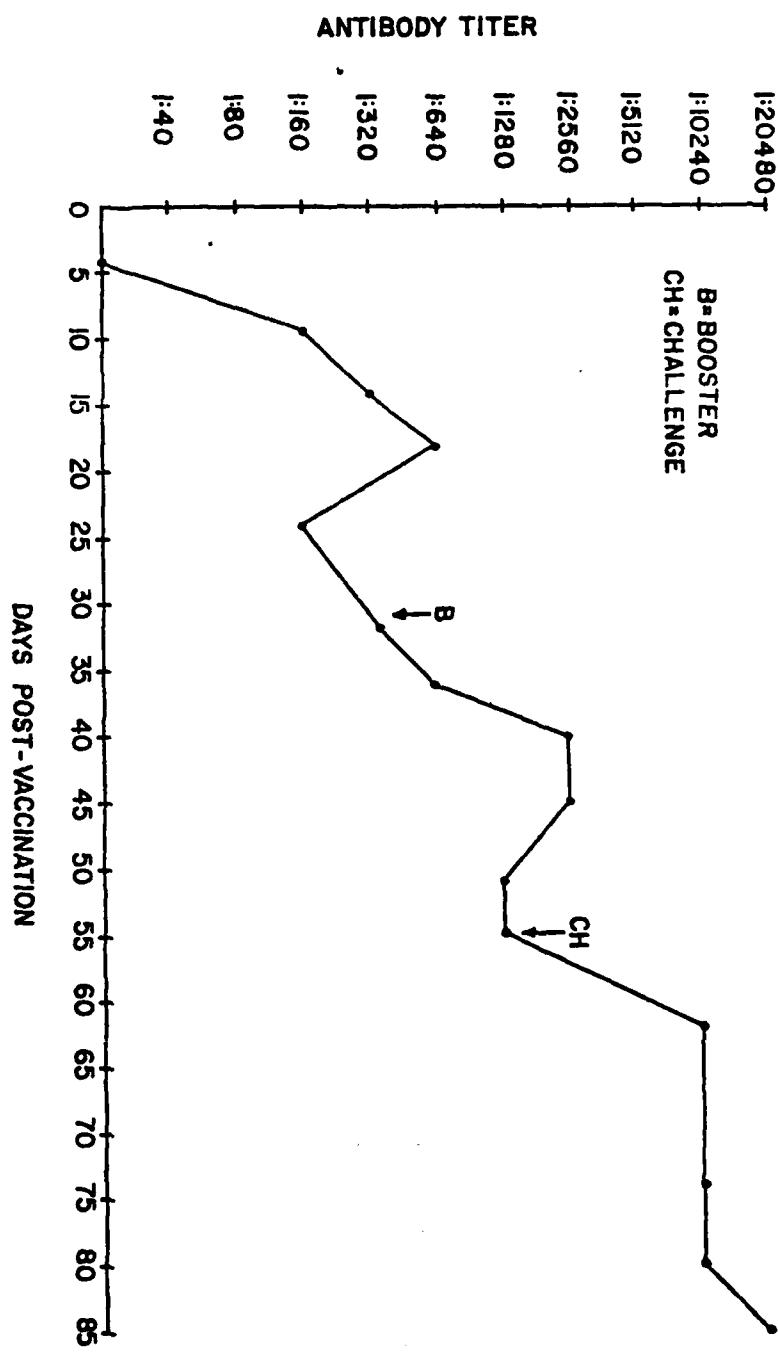


Figure 17

Figure 18



of the second dose. There was no detectable antibody response in the IFA test following inoculation of the first vaccine dose.

Challenge of the 3 non-vaccinated dogs produced a sharp and steady rise of antibody titer which reached the highest level of 1:2560 at approximately 30 days after challenge. An example of control dog 650 is given in Figure 19.

Indicators of protective immunity. Dogs of the 2 vaccinated groups appeared to have been protected against challenge as indicated by their post challenge recordings of body temperature, hematocrit levels, parasitemia, and their general clinical condition. Body temperature of all dogs remained at normal levels, hematocrits decreased from an average of 35% to 33% during a transitory one-week period. At no time was there parasitemia noted, and the animals consumed their food well and appeared clinically in good health.

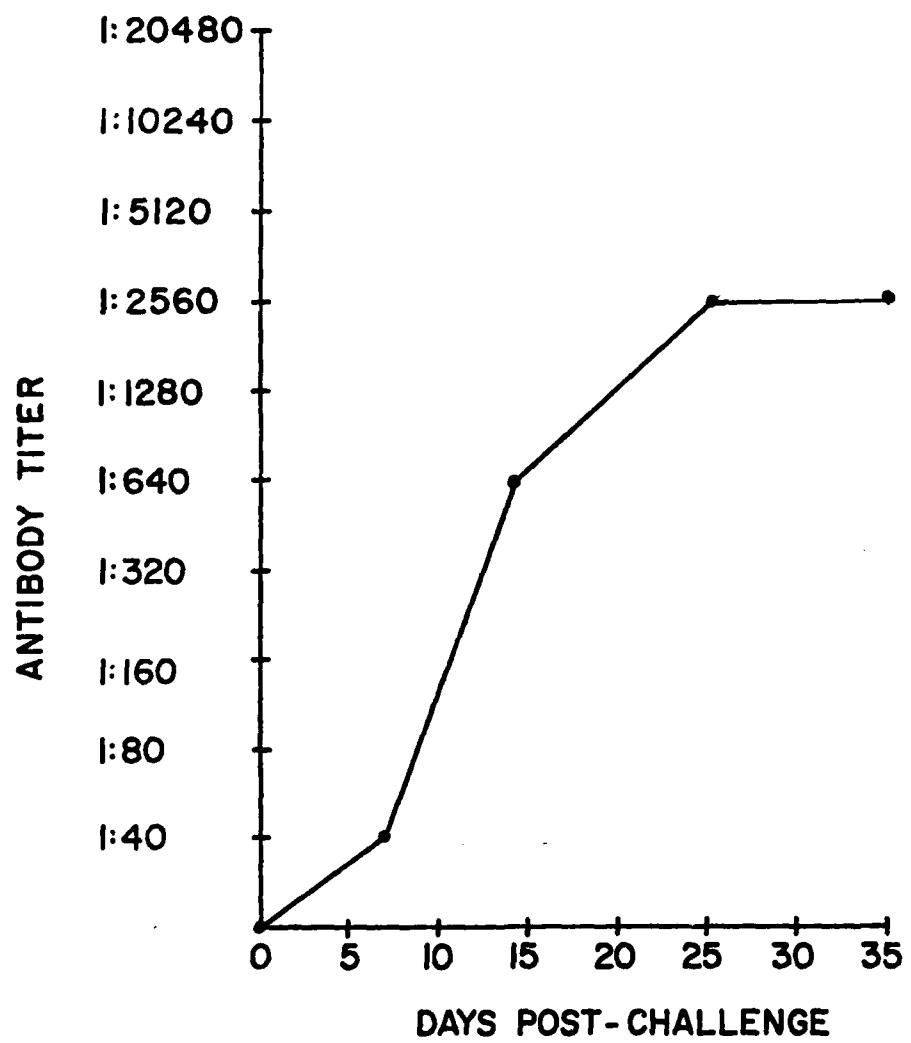
All three control animals developed severe fever ranging from 102° to 104.5° F, hematocrit levels dropped from an average of 36% to an average of 23%, at least one dog showed hemoglobinuria, and B. canis was detected in erythrocytes of the other 3 dogs.

Clinically, all 3 dogs showed severe anorexia and lethargy.

#### Discussion

The unique feature of the cell cultural system for propagation of B. canis is that it provided for mass production of extracellular merozoites. These parasitic forms have rarely, if ever, been observed in the blood of living animals affected by babesiosis. An obvious reason for the lack of detectable cell-free merozoites in the blood of living animals is a very rapid removal from the circulation by the defense elements of the reticuloendothelial system. It was shown that

Figure 19



similarly to findings in human malaria (Miller et al., 1975), B. canis merozoites are coated by a surface antigen (Fig. 13). Preliminary evidence in malaria indicates that this surface antigen plays a role in the attachment and penetration of erythrocytes by Plasmodium species. Consequently, it is believed that antibodies to the surface merozoite antigen are important in protection against malaria.

The present study in B. canis expands our knowledge on the surface merozoite antigen by revealing its occurrence as a free substance in the supernatant culture medium. The finding may have future practical ramification as it provides a source of potential immunogens free of the parasite.

By molecular sieve chromatography using Sephadex G-200 column, soluble B. canis antigens from the serum of acutely infected dogs co-eluted with albumin and hemoglobin indicating a molecular range of 60,000 to 70,000. In contrast, soluble cell culture antigens (S12000) eluted in the IgM peak indicating a molecular weight of 900,000. The homogeneity of the antigens found in acute serum is in contrast to the relative heterogeneity of the antigens derived from cell culture as shown by the number of precipitin lines in the Ouchterlony test. It is possible that all the antigens are released in vivo but some are more resistant to host enzyme degradation than others. There may also be a selective clearance of large molecular weight moieties by the reticuloendothelial system since, as a rule, large complexes have high affinity for macrophages. Serum from the acutely infected animals contained both antigen and antibody, and the IgM and IgG peaks in the acute G-200 serum profile are enriched with specific antibody. The excess antibody is

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Miller, L. H., Aikawa, M. and Dvorak, J. A.: Malaria (Plasmodium knowlesi) Merozoites: Immunity and the Surface Coat. J Immunol, 114 (1975): 1237-1242.

likely to competitively inhibit precipitation in the zone of antibody excess, thereby making it difficult to demonstrate small quantities of antigen in acute serum. The absence of small molecular weight antigen in S12000 could also be due to complex formation under in vitro conditions, leading to a shift from low to predominantly high molecular weight complexes. It was observed that Giemsa-stained merozoites often appeared in massive aggregates and it is possible that merozoite-derived antigens would also tend to clump together under in vitro conditions thereby forming large molecular weight antigenic moieties.

The S12000 antigens were thermolabile at 100° C for 15 minutes and sensitive to reducing agents. This suggests that proteins with disulphide bonds form a major component of the antigenic moiety.

Both soluble and corpuscular B. canis cell culture-derived antigens in the presence of Quil A induced a rapid primary immune response. Secondary response following a booster antigen dose was manifested by a rapid and considerable increase in the antibody titer as detected by the indirect fluorescent antibody test. In this regard, secondary immune response indicates a greater amplification of memory cells. Coincident with high antibody titer, vaccinated dogs fail to develop any parasitemia or any other symptom of canine babesiosis. While these data must be considered preliminary mainly because of a limited number of dogs used, it appears that a larger vaccination experiment of dogs with cell culture-derived antigens fortified by Quil A adjuvant may be a feasible immunoprophylactic endeavor. Such an experiment is now being planned in collaboration with LTC David Huxsoll of the U.S. Army Medical Component in Kuala Lumpur, Malaysia.

Summary

Babesia canis derived from parasitemic dogs was propagated in primary cell culture in modified RPMI 1640. Relative numbers of extracellular and intracellular parasites were monitored daily. The maximum intracellular parasitemia occurred at 24 hours of cultivation. Extracellular Babesia forms reached maximal levels at 48 hours. Stages of erythrocytic invasion and different phases of replication of the organism were demonstrated by light and electron microscopy. Ultra-structural studies demonstrated the presence of a surface coat on the extracellular (merozoite) forms of B. canis.

Soluble Babesia antigens were isolated from the cultures by ammonium sulphate precipitation and molecular sieve chromatography on Sephadex G-200. The antigens reacted specifically with immune serum obtained from dogs recovered from B. canis infection when tested in gel diffusion. Antigens derived from primary cell culture appeared to be heterogeneous but were all eluted in the first peak of Sephadex G-200 indicating a molecular weight around 900,000. Antigens obtained from serum of a dog suffering from acute babesiosis had limited heterogeneity and co-eluted with albumin corresponding to a molecular weight of 60,000 to 70,000. The antigenicity was destroyed by heating at 100° C for 15 min and was sensitive to 0.1 M 2-ME. The possible role of the reticuloendothelial system in limiting the heterogeneity of B. canis antigens in vivo is postulated.

Immunogenic characteristics of the cell culture-derived antigens were assayed by vaccination of susceptible dogs. Vaccinated dogs demonstrated a strong humoral antibody response and resisted challenge with virulent

B. canis blood in that they never displayed clinical babesiosis, and no parasites were ever detected in their peripheral blood. Unvaccinated controls succumbed to babesiosis characterized by parasitemia, anemia and general lethargy. It is suggested that B. canis propagated in vitro may provide a potential source of a vaccine against canine babesiosis.

C. Research Accomplished Under Objective 3

Study of the serologic relationship between Ehrlichia canis and human sennetsu rickettsiosis and canine rickettsiosis.

The study was initiated as a result of a preliminary finding that Ehrlichia canis, the causative agent of canine ehrlichiosis (tropical canine pancytopenia - TCP) and Rickettsia sennetsu, the causative agent of human sennetsu rickettsiosis, were morphologically and antigenically similar. Until the present, it was believed that each of these agents were morphologically and antigenically unique with reference to other common rickettsiae. Sennetsu rickettsiosis is still a relatively obscure human disease with little, if any, information regarding the mode of transmission. Simple and accurate serodiagnostic methods are also lacking and accordingly little knowledge is available on the epidemiology of the disease and its geographic distribution.

Because of a great amount of technical knowledge and experience gained from the study of E. canis in a long-term joint research effort of scientists from the WRAIR and this laboratory, and because of the apparent similarity of E. canis to R. sennetsu, it was felt advantageous to undertake studies toward clarification of the sennetsu rickettsiosis syndrome.

This report is presented in two parts: The first portion concerns an expansion of the study of cross-serologic relationship between E. canis and E. sennetsu (see 1977 Report). The second portion describes the establishment of R. sennetsu cell cultures in our laboratory. Examination of the cross-serologic relationship between these agents has been done in Urbana and in the

laboratories of Dr. Hobuyoshi Tachibana at the Miyazaki Medical School, Kiyotake, Miyazaki, Japan. Indirect fluorescent antibody tests were used at both locations. In Urbana, E. canis antigen was tested against positive human sera received from Dr. Tachibana, while in Japan, R. sennetsu antigen was tested against positive canine sera provided to Dr. Tachibana by COL David L. Huxsoll of Kuala Lumpur, Malaysia. Results of the latter study are preliminary and are based upon a joint examination by Drs. Huxsoll and Tachibana during their recent meeting in Dr. Tachibana's laboratory.

1. Further studies on cross-serologic relationship between *Ehrlichia canis* and *Rickettsia sennetsu*: *Ehrlichia canis*, the type species of the genus *Ehrlichia*, family Rickettsiaceae, is the causative agent of canine ehrlichiosis, an acute tick-borne disease of domestic and wild canidae (Philip, 1974). The disease is manifested by progressive pancytopenia, particularly thrombocytopenia, anorexia, emaciation, dehydration and increased body temperature. A fulminating form of the disease manifested by epistaxis which caused severe losses among military dogs in Viet Nam during 1968 and 1970 is referred to as tropical canine pancytopenia (TCP) (Huxwoll et al., 1970). During the acute stage of infection, the organism occurs in the cytoplasm of circulating monocytes in the form of a "morula," an inclusion body consisting of many small "elementary" bodies. Inclusions occur within a membrane-lined

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Philip, C. B.: *Ehrlichiae*. In Bergey's Manual of Determinative Bacteriology. R. E. Buchanan and N. E. Gibbons, eds., 8th ed. (1974) 893-895. Williams and Wilkins, Baltimore, MD.

Huxsoll, D. L., Hildebrandt, P. K., Nims, R. M., and Walker, J. S.: Tropical Canine Pancytopenia. JAVMA, 157 (1970): 1627-1632.

vacuole which separates individual organisms from host cell cytoplasm. The organisms are bound by an outer trilaminar cell wall and an inner trilaminar plasma membrane (Hildebrandt, et al., 1973).

Development of a cell culture method for propagation of E. canis (Nyindo, 1971) has led to the development of an indirect fluorescent antibody (IFA) test for detection and quantitation of anti-E. canis antibodies (Ristic et al., 1972). The test proved to be an accurate and specific means for detecting dogs clinically and subclinically infected with E. canis. Extensive cross-serologic studies with sera to various canine pathogens and 8 rickettsiae produced no evidence of antigenic relationship between E. canis and any of these agents.

Rickettsia sennetsu is the causative agent of human sennetsu rickettsiosis, or "infectious mononucleosis" in western Japan, differentiating it from mononucleosis in other parts of Japan and other countries. The disease, which is widely distributed in western Japan, is characterized by fever, general lymph node

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Hildebrandt, P. K., Conroy, J. E., McKee, A. E., Nyindo, M. B. A., and Huxsoll, D. L.: Ultrastructure of Ehrlichia canis. Infect Immun., 7 (1973): 265-271.

Nyindo, M. B. A., Ristic, M., Huxsoll, D. L., and Smith, A. R.: Tropical Canine Pancytopenia: In vitro Cultivation of the Causative Agent-Ehrlichia canis. AJVR, 32 (1971): 1651-1658.

Ristic, M., Huxsoll, D. L., Weisiger, R. M., Hildebrandt, P. K., and Nyindo, M. B. A.: Serologic Diagnosis of Tropical Canine Pancytopenia by Indirect Immunofluorescence. Infect Immun., 6 (1972): 226-231.

enlargement, and absolute increase in normal and atypical lymphocytes (Misao, 1966). No information is available regarding the means of transmission of the disease although arthropod vectors are suspected. The agent was first isolated in 1953 from the blood, lymph nodes and bone marrow fluid of a patient who showed the typical symptoms of infectious mononucleosis (Misao and Kobayashi, 1954). The organism was confirmed to be the etiologic agent of the disease by experimental infection in human volunteers. Although the natural cycle of the organism has not been fully established, the agent has the general morphological and biological properties of rickettsiae and thus was named Rickettsia sennetsu in 1956 (Misao et al., 1957).

While the clinical and pathological symptoms of sennetsu rickettsiosis are similar to those of infectious mononucleosis, the sera of patients with mononucleosis in the U.S. gave negative reaction when tested against R. sennetsu by the immunofluorescent antibody test (Misao, 1966). Microscopic and electron microscopic studies of R. sennetsu showed that the agent morphologically differs from other classic rickettsiae. Unlike the latter organisms, R. sennetsu does not occur freely in the host cell cytoplasm but rather single or

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Misao, T.: Rickettsial Disease in Western Japan. J Japan Assoc Infect Dis, 40 (1966): 71-73.

Misao, T. and Kobayashi, Y.: Studies on Infectious Mononucleosis. I. Isolation of Etiologic Agent from Blood, Bone Marrow and Lymph Node of a Patient with Infectious Mononucleosis by Using Mice. Tokyo Iji Shinshi, 71 (1954): 683-686.

Misao, T., Kabayashi, Y. and Shirakawa, M.: La Mononucléose Infectious (Fievre Ganglionnaire). Sang, 28 (1957): 785-805.

multiple forms of the organism are contained in a membrane-like vacuole (Anderson et al., 1965). These structures closely resemble morulae of E. canis and can similarly be detected in the cytoplasm of circulating blood monocytes. Although R. sennetsu exhibits certain rickettsial properties, no serologic relationship to other rickettsial agents has been established, thus until its biological properties are better known, it is regarded as a species incertae sedis (Weiss and Moulder, 1974).

Because of a close morphologic resemblance between R. sennetsu and E. canis, and because of their apparent antigenic uniqueness with reference to other rickettsiae, we decided to investigate the serologic relationship between these two agents.

a. Materials and Methods

Antigens and serologic tests: Ehrlichia canis used in this study was recovered in Florida in 1969 from blood of a German shepherd dog with signs of TCP (Huxsoll et al., 1970). The organism propagated by an in vitro technique (Nyindo et al,

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Anderson, D. R., Hopps, H. E., Barile, M. F. and Bernheim, B. C.: Comparison of the Ultrastructure of Several Rickettsiae, Ornithosis Virus, and Micoplasma in Tissue Culture. J Bact, 90 (1965): 1387-1404.

Weiss, Emilio and Moulder J. W.: Genus Rickettsia. In Bergey's Manual of Determinative Bacteriology. R. E. Buchanan and N. E. Gibbons, eds., 8th ed, (1974): 883-885, Williams and Wilkins, Baltimore, MD.

Huxsoll, D. L., Hildebrandt, P. K., Nimms, R. M., Amyx, H. L., and Ferguson, J. A.: Epizootiology of Tropical Canine Pancytopenia. J Wildlife Dis, 6 (1970): 220-225.

Nyindo, M. B. A., Ristic, M., Huxsoll, D. L. and Smith, A. R.: Tropical Canine Pancytopenia: In vitro Cultivation of the Causative Agent - Ehrlichia canis. AJVR, 32 (1971): 1651-1658.

1971) in canine blood monocytes served as an antigen in the indirect fluorescent antibody (IFA) test (Ristic et al., 1972). Antisera to human and canine globulins were produced in rabbits. Preparation of gamma globulins and methods of inoculation are described elsewhere (Goldman, 1968).

The gamma globulin was extracted from the immune rabbit sera by precipitation with 15% (w/v) sodium sulfate solution. The precipitate was dissolved in a volume of 0.15 M sodium chloride solution equal to 20% of the original serum volume and dialyzed overnight against 0.175 M sodium phosphate buffer (pH 6.3) at 3° C. The dialyzed protein was separated in diethylaminoethyl cellulose equilibrated in 0.175 M phosphate buffer (pH 6.3). The protein concentration was adjusted to 10 mg/ml and labeled with fluorescein isothiocyanate (0.033 mg/mg protein) dissolved in 0.5 M carbonate buffer solution (pH 9.5). The mixture was stored at 22° C for 2 hr, then passed through a Sephadex G-25 column to remove free fluorescein isothiocyanate. The conjugate was absorbed for 30 min at 22° C with lyophilized bovine spleen powder in a ratio of 1 ml of conjugate to 10.0 mg of powder. The conjugate was stored in 0.25 ml samples at -65° C.

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Ristic, M., Huxsoll, D. L., Weisiger, R. M., Hildebrandt, P. K., and Nyindo, M. B. A.: Serologic Diagnosis of Tropical Canine Pancytopenia by Indirect Immunofluorescence. *Infect Immun*, 6 (1972): 226-231.

Goldman, M.: Fluorescent Antibody Methods. Academic Press, Inc., New York, NY, 1968.

Sera from 5 patients with sennetsu rickettsiosis were pooled, gamma globulins extracted, conjugated with fluorescein isothiocyanate and then used to stain E. canis by means of a direct fluorescent antibody (FA) method. Similar control preparations were made from sera of apparently normal human beings.

The Miyayama strain, prototype of R. sennetsu, isolated in 1953 (Misao and Kabayashi, 1954) and kept in the laboratory by passage in mice was used as an antigen. Spleens of mice experimentally infected with R. sennetsu served as a source of particulate antigen used in the IFA test.

Test sera: All 5 human patients affected with R. sennetsu were males varying from 17 to 59 years of age. They were residents of Fukuoka and were treated for sennetsu rickettsiosis in the teaching hospital of Kyushi University during September through November of 1976. Typical signs of the disease were intermittent fever ranging between 38° - 40° C, headache and back pain, sore throat, skin rash, lymphadenopathy and occasionally hepatomegaly. Sera for serologic study were obtained 12-15 days after onset of the disease. Sera 1-4 were delivered to the University of Illinois for examination in the E. canis IFA test, frozen in dry ice and serum No. 5 was received as a lyophilized sample.

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Misao, T. and Kobayashi, Y.: (Studies on Infectious Mononucleosis. I. Isolation of Etiologic Agent from Blood, Bone Marrow and Lymph Node of a Patient with Infectious Mononucleosis by Using Mice. Tokyo Iji Shinshi, 71 (1954): 683-686.

Six sera from dogs convalescing from experimentally induced infection with E. canis were sent as frozen samples from the U.S. Army Medical Research Unit at Kuala Lumpur, Malaysia, to Kyushu University for examination in the R. sennetsu IFA test. Preinfection sera of these dogs served as controls in the test.

Antisera to a variety of rickettsiae, as well as sera from apparently normal human beings, were used to ascertain specificity of the E. canis IFA test.

b. Results

Results of examination in the E. canis IFA test of sera from 5 human patients infected with R. sennetsu are given in Table 1. Serum No. 5 which was received in lyophilized form reacted at 1:80 titer while the remaining 4 sera reacted at 1:160 titer. None of the 8 sera from apparently normal human beings reacted at 1:5 serial serum dilution. In a homologous IFA test using R. sennetsu antigen, serum No. 3 showed titer of 1:512 and the remaining 4 sera reacted at 1:128 titer. None of the 14 specific antisera to 10 common rickettsiae reacted at 1:5 dilution against E. canis (Table 2).

The appearance of canine monocyte infected with E. canis following staining with fluorescein conjugated (direct method) human anti-R. sennetsu immunoglobulins is shown (Fig. 1). Multiple intracytoplasmic morulae stained brilliant yellow are clearly visible by the method. Such E. canis inclusions were not stained with similar preparations of serum globulins from apparently normal human beings.

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Table 1

Serologic cross-reaction between human anti-Rickettsia sennetsu sera and Ehrlichia canis antigen using the indirect fluorescent antibody (IFA) test

Human Serum	E. canis						<u>R. sennetsu</u> titer
	Reaction at serum dilution						
	1:5	1:10	1:20	1:40	1:80	1:160	
1	2+	2+	2+	1+	±	±	1:128*
2	2+	2+	2+	1+	1+	±	1:128
3	2+	2+	1+	1+	1+	1+	1:512
4	2+	2+	1+	1+	±	±	1:128
5	2+	2+	1+	1+	±	-	1:128
8 normal human sera	-	-	-	-	-	-	-

Intensity of reaction evaluated by comparison within system rather than between systems.

\*Reciprocal of the final serial serum dilution showing reaction in the test.

- = organism not visible.

± = organism faintly identifiable.

1+ = good staining of the organism.

2+ = strong staining of the organism.

Table 2

Specificity of *Ehrlichia canis* indirect fluorescent antibody (IFA) test.

Agent	Immune animal species	Homologous IFA titer	IFA for <u><i>E. canis</i></u> (1:5 serum dilution)
<u><i>Rickettsia tsutsugamushi</i></u>	human	> 1:200 (Karp strain) > 1:200 (Gilliam strain)	-*
<u><i>Rickettsia rickettsii</i></u>	human	> 1:400 (Karp strain) > 1:800 (Gilliam strain)	-
<u><i>Rickettsia typhimurium</i></u>	human	> 1:640	-
<u><i>Rickettsia tsutsugamushi</i></u>	guinea pig	> 1:2,560	-
<u><i>Rickettsia rickettsii</i></u>	guinea pig	> 1:20,000	-
<u><i>Rickettsia canada</i></u>	guinea pig	> 1:20,000	-
<u><i>Rickettsia burnetti</i></u>	guinea pig	> 1:20,000	-
<u><i>Rickettsia mooseri</i></u>	guinea pig	> 1:20,000	-
<u><i>Rickettsia akari</i></u>	guinea pig	> 1:20,000	-
Psittacosis agent	guinea pig	> 1:20,000	-
Tatlock agent ( <i>rickettsia</i> -like agent of guinea pig origin	guinea pig	> 1:20,000	-

\*No reaction.

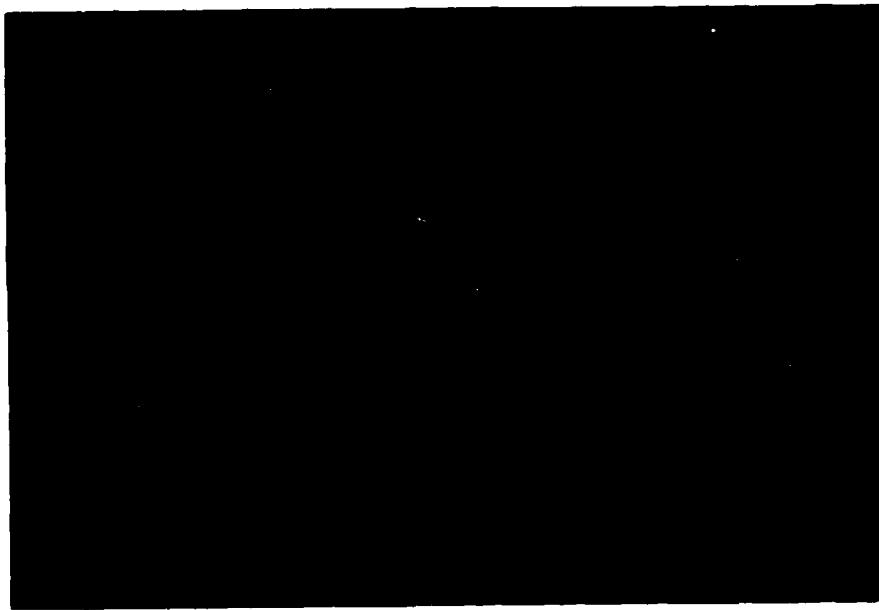


Figure 1. The appearance of canine monocyte infected with E. canis following staining with fluorescein-conjugated human anti-R. sennetsu immunoglobulins. Note multiple intracytoplasmic morulae (arrow) X 1800.

Results of examination by the R. sennetsu IFA test of 6 canine sera collected before and after infection with E. canis are given in Table 3. None of the preinfection sera reacted at titer of 1:2. All post infection sera reacted at 1:1024 to 1:2048 titers. Homologous titers of these sera were slightly lower, ranging between 1:320 to 1:1280.

c. Discussion

A good degree of cross-serologic activity between R. sennetsu and E. canis and their specific sera was demonstrated in this study. In fact, anti-E. canis sera reacted at slightly higher titer with R. sennetsu antigen than with the homologous antigen. This disparity, however, may be caused by a difference in the IFA system used with R. sennetsu compared with

Table 3

Serologic cross-reaction between canine anti-*Ehrlichia canis* sera and  
*Rickettsia sennetsu* antigen using indirect fluorescent antibody (IFA) test

Canine serum	IFA titer	
	<u><i>E. canis</i></u>	<u><i>R. sennetsu</i></u>
1	1:640*	1:2048
2	1:320	1:1024
3	1:1280	1:1024
4	1:320	1:512
5	1:640	1:2048
6	1:320	1:1024
6 normal sera	Neg at 1:5	Neg at 1:2

\*Reciprocal of the final serial serum dilution showing reaction in the test.

that of E. canis. In the R. sennetsu IFA test a cell-free and relatively pure antigen is used, while in the E. canis system the antigen is contained in cultured monocytes derived from peripheral blood. In the direct fluorescent antibody test illustrated in Figure 1, conjugated anti-R. sennetsu globulins depicted a complete structural configuration of E. canis morulae, suggesting that this conjugate contained optimal concentrations of specific antibody to induce intense and wide-spectrum staining.

Evidence of serologic relationship between R. sennetsu and E. canis is a significant finding for several reasons. While the 2 agents are pathogens of the two widely separated animal species, man and dog, their relationship to the host cell is similar and strikingly different from the other rickettsiae. Most of the rickettsiae studied occur free in the cytoplasm of the host cell and are bound by a cell wall and plasma membrane. In contrast, R. sennetsu and E. canis are not found free in the host cell cytoplasm but occur in clusters enclosed by a membrane of host cell origin (Anderson et al., 1965; Hildebrandt et al., 1970). In morphological terms, such structures resemble inclusions typical of lymphogranuloma-venereum-trachoma (LGVT) group of agents. No life cycle characteristic of LGVT agents, however, was demonstrated

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Anderson, D. R., Hopps, H. E., Barile, M. F., and Bernheim, B. C.: Comparison of the Ultrastructure of Several Rickettsiae, Ornithosis Virus, and Micoplasma in Tissue Culture. *J Bact*, 90 (1965): 1387-1404.

Hildebrandt, P. K., Conroy, J. D., McKee, A. E., Nyindo, M. B. A., and Huxsoll, D. L.: Ultrastructure of Ehrlichia canis. *Infect Immun.*, 7 (1973): 265-271.

with either R. sennetsu or E. canis. Like the majority of rickettsiae, both agents multiply by binary fission. Thus, from the morphological host cell-parasite point of view the 2 agents display unique characteristics among rickettsiae. This morphologic uniqueness of the 2 agents is further amplified by a lack of serologic relationship between them and other major rickettsiae including the LGVT group of agents (Tanaka and Nanoaka, 1961).

Sennetsu rickettsiosis is an important human disease, however, its distribution based upon present information seems to be limited to western Japan. Knowledge regarding certain aspects of this disease, particularly its mode of transmission, source of infection, epidemiology and protective immunity, are relatively limited. Infections with E. canis and the occurrence of TCP are, on the other hand, world-wide with a great amount of knowledge available regarding host-parasite-vector relationship. In addition, considerable data have been accumulated on the pathogenesis, chemotherapy and chemoprophylaxis and the mechanism of protective immunity in infection caused by E. canis. Thus, experience gained from studies of E. canis and TCP is an invaluable foundation for further pursuance of the true biologic relationship of the 2 agents and the diseases they cause.

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Tanaka, H. and Nanoaka, M.: Ultrastructure and Taxonomy of Rickettsia sennetsu (the Causative Agent of "sennetsu" or Infectious Mononucleosis in West Japan) as Studied with the Electron Microscope. Ann Report Inst Virus Res., Kyoto University, 4 (1961): 67-82.

d. Summary

The serologic relationship between Rickettsia sennetsu, the etiologic agent of human sennetsu rickettsiosis in Western Japan, and Ehrlichia canis, the agent of canine ehrlichiosis and tropical canine pancytopenia (TCP), has been demonstrated. Using the indirect fluorescent antibody (IFA) test, the two agents cross-reacted with convalescent canine and human sera, respectively. The degree of cross-reactivity was high, judged by homologous and heterologous titers. In the direct fluorescent antibody (FA) test, immunoglobulins from 5 patients with sennetsu rickettsiosis stained E. canis morulae contained in infected canine monocytes.

The significance of this finding was discussed in relation to the morphologic uniqueness of the 2 agents and their lack of serologic relations with other major rickettsial agents.

2. Propagation of Rickettsia sennetsu in human blood monocyte cell cultures: Preliminary studies on R. sennetsu as described in the first portion of the report under Objective 3 dealt with demonstration of cross-serologic reactivity between E. canis antigens and antibodies in sera of patients recovered from sennetsu rickettsiosis. An expansion of the research program required that we secure R. sennetsu for study in our laboratory. After considerable inquiries, we learned that no laboratory in the U.S. has ongoing research with this agent and that the only possible source is the American Type Culture Collection (ATCC). The organism was deposited in the ATCC in 1972 by Mrs. Hope E. Hopps of the National Institutes of Health. The organism was on deposit

in 1 ml vials as homogenates of infected African green monkey kidney (AGMK) continuous cell line BSC-1. Since its deposit, the culture was maintained in a Reyco freezer at approximately -65° C. We purchased AGMK cell line<sup>a</sup> and established growth in our laboratory.

a. Attempts to propagate R. sennetsu in BSC-1 cell line: The BSC-1 cells were grown in Eagle's minimum essential medium (MEM) with Earle's salts supplemented with 2% fetal bovine serum, 1% L-glutamine (400 mM) and Hepes buffer. No antibiotics were used in the medium. Cell monolayers were developed in 30-ml plastic tissue culture flasks<sup>b</sup> and Leighton<sup>c</sup> tissue culture tubes (16 x 95 cm), each provided with 0.8 by 4.0 cm coverslips.<sup>c</sup>

Cell culture monolayer in an intense mitotic state and with 70 to 80% sheet layer formed were used for infection with R. sennetsu.<sup>c</sup> Rickettsia sennetsu seed received from ATCC was thawed in a 37° C water bath and rapidly diluted 1:5 using the above-described medium supplemented with 10% fetal calf serum. Cell monolayers in flasks and Leighton tubes were inoculated with 2 or 0.5 ml of the diluted seed material and allowed to stand 2 hours for absorption of the organism. The medium containing 10% fetal

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<sup>a</sup>Microbiological Associates, Biggs Ford Road, Walkersville, MD

<sup>b</sup>Falcon Plastics, Division of Bio-Quest, Los Angeles, CA.

<sup>c</sup>Belco Biological Glassware, Vineland, NJ.

calf serum was then added and cultures maintained at 37° C. Cultures were refed only when the media turned alkaline.

To ascertain the presence of R. sennetsu, coverslips were removed on different days, ranging from 6 to 20 days of culture. The coverslips were split in half. One half was stained with Giemsa technique, while the other was fixed in acetone for 10 minutes and stained with fluorescein conjugated anti-R. sennetsu human serum. The test was controlled using both conjugated normal human serum and uninfected cell cultures.

As an additional measure to ascertain the presence of R. sennetsu in our cell cultures, we prepared homogenates represented by supernatant fluids and monolayers of cell cultures infected 16 days earlier. Quantities of 0.25 ml of these homogenates were injected intraperitoneally into 20 laboratory mice. Groups of 5 mice were euthanized every 6 days, examined for gross pathologic changes and Giemsa stained films made from their spleens, peritoneal macrophages and blood.

The above-described efforts to isolate and grow R. sennetsu from the seed material on deposit in the ATCC has been done two times with each of the 2 shipments received. Based upon microscopic examination of the cultures and upon the infectivity status of inoculated mice, there was no indication that we propagated R. sennetsu using the seed received from ATCC. It was at this stage of efforts to establish R. sennetsu in our laboratory that we resorted to using cultures of human peripheral blood monocytes.

b. Rickettsia sennetsu in monocyte cell cultures

1) The organism: Rickettsia sennetsu isolated from a patient by Misao and Kobayashi in 1953 (Misao and Kobayashi, 1955; Misao, Kobayashi, and Shirakawa, 1957) was used in the present study. The organism was propagated in African green monkey kidney cells (BSC-1) and its ultrastructural properties examined by a research team of the U.S. National Institutes of Health (Anderson et al., 1965). Mrs. H. E. Hopps, a member of that research team, donated samples of the agent grown in BSC-1 cells for deposit in the laboratory of the American Type Culture Collection in 1972.

For studies in our laboratory, the organism was received frozen in dry ice. For inoculation of monocyte cell cultures, the material was thawed by rapid immersion in a 37° C water bath, diluted 1:5 with Eagle's minimum essential medium with Earle's salts supplemented with 10% human serum.<sup>a</sup>

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Misao, T. and Kobayashi, Y.: Studies on infectious mononucleosis. I. Isolation of Etiologic Agent from Blood, Bone Marrow and Lymph Node of a Patient with Infectious Mononucleosis by using mice. Tokyo Iji Shinshi, 71 (1954); 683-686.

Misao, T., Kabayashi, Y. and Shirakawa, M.: La Mononucléose Infectieuse (Fièvre ganglionnaire). Sang 28 (1957): 785-805.

Anderson, D. R., Hopps, H. E., Barile, M. F. and Bernheim, B. C.: Comparison of the Ultrastructure of Several Rickettsiae, Ornithosis Virus, and Micoplasma in Tissue Culture. J Bact., 90 (1965): 1387-1404.

<sup>a</sup>Microbiological Associates, Biggs Ford Road Road, Walkersville, MD.

2) Monocyte cultures: A 40-ml specimen of blood was obtained from the cephalic arm vein of our research team volunteers at the university infirmary. The blood was collected in a 50 ml sterile syringe coated with heparin (20,000 u/ml). The needle was discarded and replaced with a new sterile plastic-covered needle. Ten ml of a 0.25% dextran sulfate (MW 500,000) solution in normal saline warmed to room temperature was then added to the blood in the syringe. The syringe was placed in a vertical position (needle end up), and erythrocytes were allowed to sediment for 60 minutes at 22° C. The needle was then bent and 5 ml volumes of the plasma phase was infused into 25 cm<sup>2</sup> tissue culture flasks. The flasks were placed in a 37° C incubator and monocytes allowed to attach during a period of 3 - 5 hours. The supernatant was then poured off and 5 ml of medium consisting of Eagle's minimum essential medium with Earl's salts, supplemented with 10% human serum, and 1% L-glutamine (400 mM) was added. At 24 hours of incubation, the medium was replaced with fresh media. Thereafter, the cultures were refed every 2 to 3 days or when media turned alkaline. Within 2 weeks of incubation, monocytes increased in size considerably and formed a solid monolayer.

For infection, the media was removed and cell monolayers of each flask inoculated with 2 ml of the above-described

R. sennetsu seed material. Two hours at 37° C was allowed for absorption of the rickettsia after which time 3 ml of the media was added and cultures returned to the incubator. The supernatant of the first culture was used to infect the second culture prepared from another donor from our research team.

3) Examination of cultures for the presence of R. sennetsu:

At the time of media change, approximately 2.5 ml of the supernatant media to be replaced was poured into a round 5 ml flat-bottom glass tube. The bottom of the tube was provided with a round coverslip. The tube was centrifuged at 750 x g for 15 minutes, the supernatant discarded and the coverslip processed for staining by Giemsa or fluorescent antibody techniques.

4) Fluorescent antibody method: Sera from 5 patients recovering from sennetsu rickettsiosis were provided by Dr. Hobuyoshi Tachibana of the Miyazaki Medical School, Kiyotake, Miyazaki, Japan. The sera were pooled, gamma globulin extracted, conjugated with fluorescein isothiocyanate and then used to stain R. sennetsu in human monocytes derived from infected cell cultures. A similar preparation of fluorescein isothiocyanate conjugated gamma globulins obtained from 5 apparently normal human subjects were used as controls. The method used for extraction, purification and conjugation of gamma globulins

was described earlier (Ristic et al., 1972). For staining, coverslips were fixed in absolute acetone at 22° C for 5 minutes and allowed to dry for 15 minutes and then flooded with conjugated antiglobulins. The coverslips were placed in a humidified chamber and incubated at 37° C for 30 minutes; they were rinsed twice for 5 minutes each time in phosphate buffered saline (PBS) pH 7.2, rinsed for 5 minutes in distilled water and air dried. A drop of mounting fluid containing 9 parts of glycerin and 1 part of PBS was deposited on each coverslip and the coverslip placed in an inverted position on a microscopic slide. Permount fluid was placed along the edges of the coverslip to attach it to the slide. The slides were examined on a microscope equipped with an ultraviolet light source.<sup>d</sup>

- 5) Mice inoculation: Twenty-four white laboratory mice divided in 2 equal groups were used to ascertain infectivity of R. sennetsu propagated in monocyte cultures. Volumes of 0.2 ml of the supernatant of the 11-day cultures were inoculated intraperitoneally into each of 12 mice. The remaining 12 mice which served as controls received similar supernatant fluid from R. sennetsu-free cultures.

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Ristic, M., Huxsoll, D. L., Weisiger, R. M., Hildebrandt, P. K., and Nyindo, M. B. A.: Serologic Diagnosis of Tropical Canine Pancytopenia by Indirect Immunofluorescence. *Infect Immun*, 6 (1972): 226-231.

<sup>d</sup>Fisher Scientific Company, Fair Lawn, NJ.

Four mice of each group were euthanized every 8 days after infection. Gross pathologic observations were made and slides of peritoneal macrophages, splenic cross-sections, and blood stained by Giemsa were examined microscopically.

3. Results: Sequential examination of cell culture monocytes stained by the Giemsa technique indicated that R. sennetsu underwent specific phases of development. Rickettsiae were detected as early as 2 to 3 days after infection. At this stage, most monocytes harboring individual organisms showed slight discoloration of the cytoplasm. The organisms were relatively evenly distributed through the cytoplasm (Fig. 2). During the subsequent 2 to 3 days of incubation, the numbers of organisms per monocyte greatly increased by nearly completely saturating the cytoplasmic portion of the host cell. An infected monocyte stained by the direct fluorescent antibody method is shown in Figure 3. At about the same time of incubation, numerous cells containing organisms in the form of clusters were noted (Fig. 4). Within these clusters, however, individual organisms were easily differentiated, indicating that the special growth stage was not of an inclusion body type. However, during a period of 8 to 12 days of incubation, new growth forms of the organism were observed greatly resembling immature (initial) and mature (morulae) inclusion bodies of Ehrlichia canis in canine monocytes (Figs. 5,6). These inclusion bodies were oval and occasionally pleomorphic with individual organisms not being clearly differentiated. Whether there was a distinct vacuole membrane surrounding these bodies was difficult to determine by

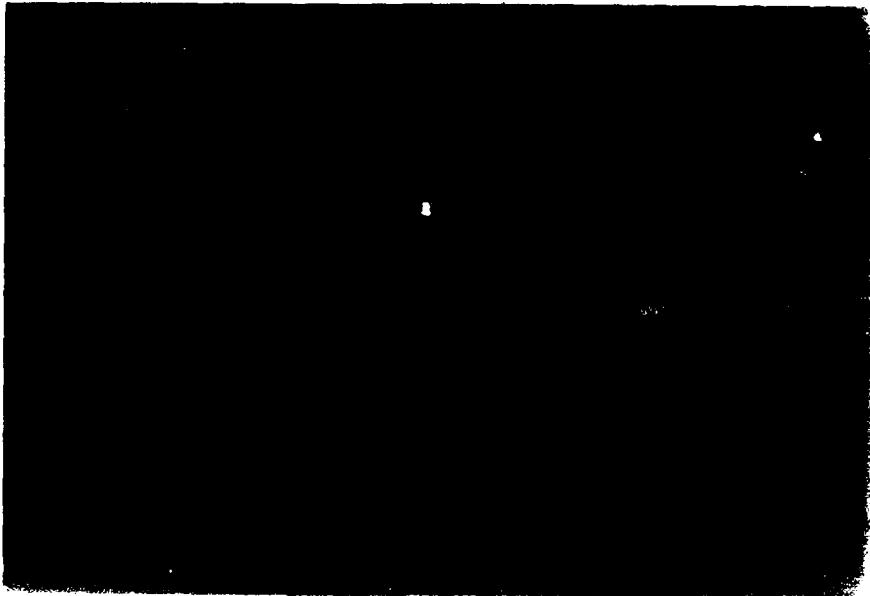


Figure 2. An early stage of development (3 days) of *R. sennetsu* with individual organism loosely distributed in the cytoplasm of cultured monocyte. Giemsa staining technique X 2100.

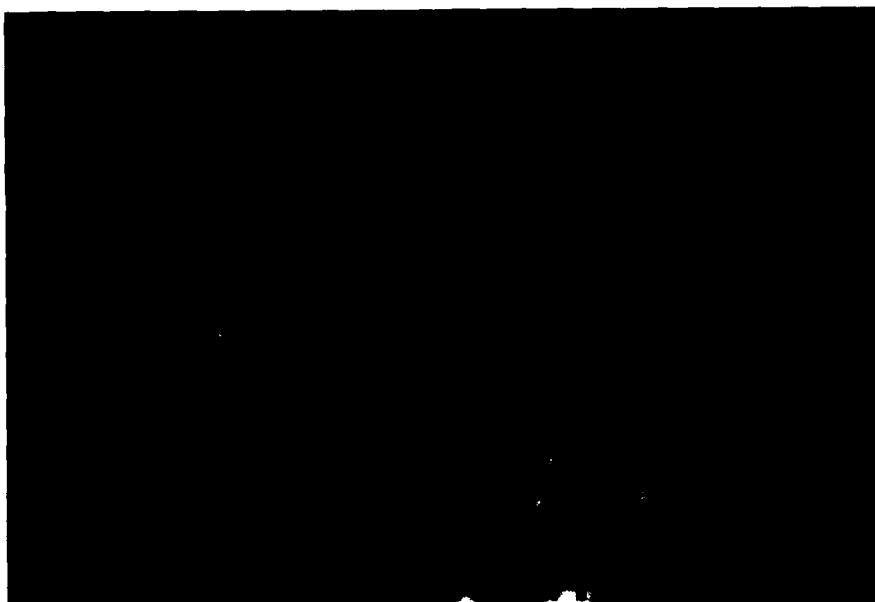


Figure 3. A monocyte retrieved from culture after 6 days of incubation. Cytoplasmic portion of the monocyte appears completely saturated with individual *R. sennetsu* organisms. Direct fluorescent antibody method X 2100.

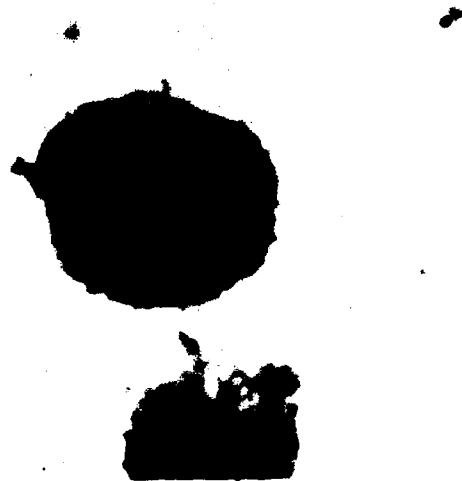


Figure 4. Rickettsia sennetsu occurring in the form of loosely packed clusters of individual organism in the cytoplasm of a cultured monocyte at 7 days of incubation. Giemsa staining method X 2100.



Figure 5. Various sizes of intracytoplasmic inclusion bodies of R. sennetsu in monocytes retrieved from cultures after 12 days of incubation. Giemsa staining technique X 2100.



Figure 6. Three larger inclusion bodies in the cytoplasm of a monocyte at 12 days of incubation. Bodies appear as densely stained oval structures clearly demarcated from each other. Giemsa staining method X 2100.

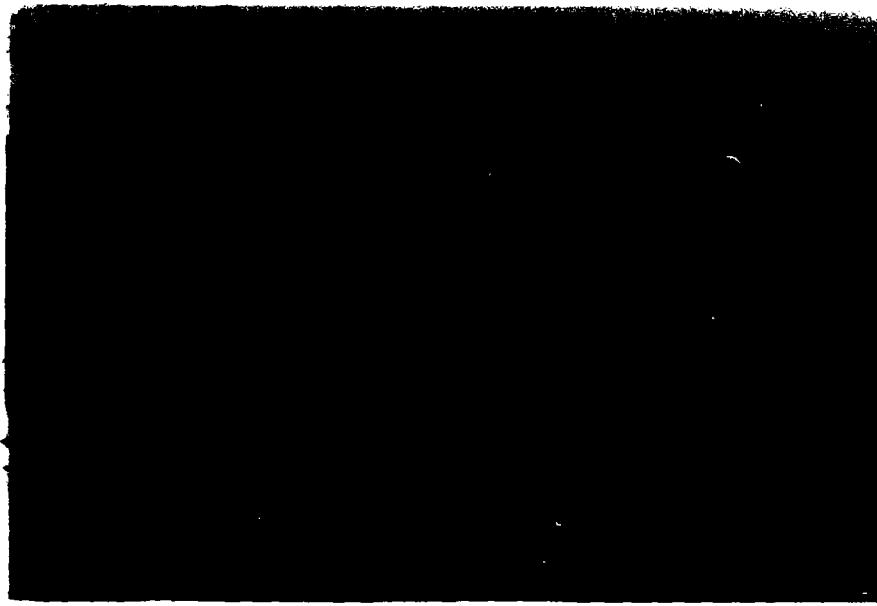


Figure 7. Numerous individual organisms and various sizes inclusion bodies dissociated from the host cell cytoplasm and occurring freely in vacuoles at 18 days of incubation. Giemsa staining method X 2100.

microscopic examination. A dozen or more morulae-like bodies were observed in the cytoplasm of some monocytes. These bodies were always clearly demarcated from each other. At this stage, many monocytes were 3 to 4 times their original size, contained 2 to 3 nuclei, and a few of these appeared in the process of mitosis. During 16 to 20 days of incubation, individual organisms and various sizes of inclusion bodies appeared dissociated from the cytoplasm and occurred freely in cytoplasmic vacuoles (Fig. 7). This growth phase was followed by apparent disintegration of monocytes and a gradual release of the organism in the media (Fig. 8). In most instances, however, organisms occurring extracellularly in the medium remained in the form of loosely packed clusters.

All 3 groups of infected mice euthanized at 8, 16 and 24 days after infection revealed the presence of the organism in their peritoneal macrophages, the blood, and splenic impression smears stained by the Giemsa method. Organisms were usually seen as a focal infection in the form of a nest-like arrangement occurring in the cytoplasm of splenic cells (Fig. 9) and similarly in the cytoplasm of peritoneal macrophages and mononuclear blood cells. Major gross tissue abnormalities included splenic hypertrophy (Fig. 10) and greatly enlarged mesenteric lymph nodes.

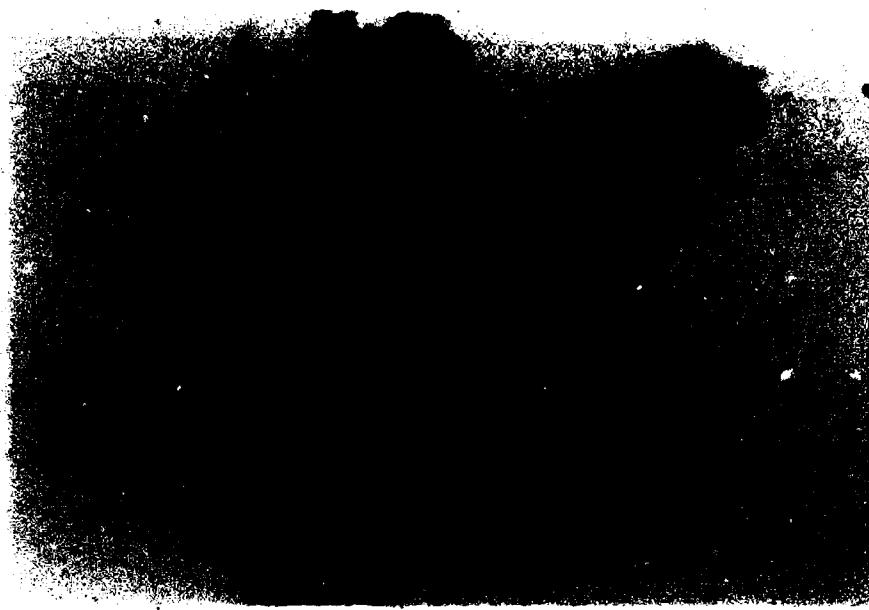


Figure 8. Final stage of growth cycle of R. sennetsu. Apparent disintegration of monocytes followed by a release of the organism in the medium at 20 days of incubation. Giemsa staining technique X 2100.

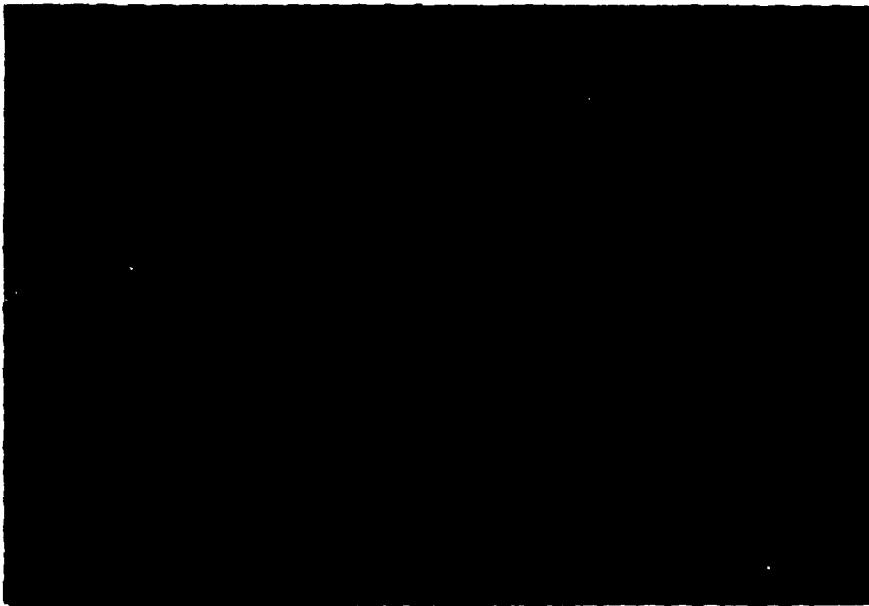


Figure 9. An impression smear of a spleen from an infected mouse. Organisms are visible in cytoplasmic vacuoles of splenic cells occurring in a nest-like arrangement (arrow). Giemsa staining method X 2100.



Figure 10. Gross appearance of spleens from a R. sennetsu infected mouse (upper) and a control mouse (lower) picture. Spleens of infected mice were usually 2 to 3 times the size of spleens from normal mice. Giemsa staining technique X 2100.

4. Discussion: The use of peripheral blood monocyte cultures has proved to be a practical means for isolation of R. sennetsu from samples stored at low temperature (-65° C) for a prolonged period of time. Under the circumstances, it is possible that the infectivity titer of this specific sample was reduced below the threshold of a minimum infectious dose needed to initiate growth of the organism in the BSC-1 cell line. Since the latter cells have been used with good results to propagate R. sennetsu, one may speculate that blood monocytes in cultures represent a more sensitive system than BSC-1 cells for isolation of the agent from samples containing minute infectious quantities of it. This reasoning is supported by previous experiences in using peripheral blood monocyte cultures toward isolation and propagation of other rickettsiae. We introduced this cultivation method for the first successful propagation of E. canis, the causative agent of canine ehrlichiosis or tropical canine pancytopenia (Nyindo et al, 1971). Since then, the method has been found useful for isolation and propagation of Neorickettsia helminthoeca from the blood of infected dogs (Brown et al, 1972), Rickettsia rickettsii from the blood of

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Nyindo, M. B. A., Ristic, M., Huxsoll, D. L. and Smith, A. R.: Tropical Canine Pancytopenia: In vitro Cultivation of the Causative Agent-Ehrlichia canis. AMJVR, 32 (1971): 1651 -1658.

Brown, J. L., Huxsoll, D. L., Ristic, M. and Hildebrandt, P. K.: In vitro Cultivation of Neorickettsia helminthoeca, the Causative Agent of Salmon Poisoning Disease. AJVR, 33 (1972): 1695-1700.

infected rhesus monkeys (Buhles et al., 1975, DeShazo et al., 1976) and more recently Rickettsia tsutsugamushi from the blood of infected silver leaf monkeys, cynomolgus monkeys and dogs (Shirai et al., 1978).

The above accumulated evidence of the apparent great sensitivity of cultured monocytes for isolation of various rickettsiae needs some elucidation of underlying mechanisms which may be contributing to the phenomenon. Blood monocytes exist in a variety of physiological stages. These differences are, in all probability, related to the effects of specific inducing agents operational in the host from which the monocytes have been derived. In case of monocytes derived from the blood of an infected host, it would be logical to speculate that these are, at least to a degree, represented by the lymphokine-stimulated macrophages. These macrophages possess enhanced microbiocidal properties (Mackaness, 1970). Thus, under the circumstances the process of rickettsial entry into the host cell would require little participation by the microbe other than the initial step of attachment (Jones, 1974).

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Buhles, W. C., Huxsoll, D. L., Ruch, G., Kenyon, R. H. and Elisberg, B. L.: Evaluation of Primary Blood Monocyte and Bone Marrow Cell Culture for the Isolation of Rickettsia rickettsii. *Infect Immun*, 12 (1975): 1457-1463.

DeShazo, R. D., Boyce, J. R., Osterman, J. V. and Stephenson, E. H.: Early Diagnosis of Rocky Mountain Spotted Fever. Use of Primary Monocyte Culture Technique. *JAMA*, 235 (1976): 1353-1355.

Shirai, A., Sankardan, V., Gan, E. and Huxsoll, D. L.: Early Detection of Rickettsia tsutsugamushi in Peripheral Monocyte Cultures Derived from Experimentally Infected Monkeys and Dogs. *SE Asian J Trop Med Pub Hlth*, 9 (1978): 11-14.

Mackaness, G. B.: The Mechanisms of Macrophage Activation. In *Infectious Agents and Host Reactions*. S. Mudd, ed. (1970) pp 62-67. W. B. Saunders Co., Philadelphia, PA.

Jones, T. C.: Macrophages and Intracellular Parasitism. *J. Reticuloendothelial Soc*, 15 (1974): 439-450.

Once in the macrophage, rickettsiae must possess a mechanism enabling them to evade lysosomal action. Such a mechanism is easily conceived for the rickettsiae, i.e., E. canis, R. sennetsu, C. burnetii, and Chlamydiae, which remain separated from the lysosomes and eventually multiply in the vacuoles. For members of the genus Rickettsia, which are deprived of a similar vacular barrier Weise, (1973) suggested that they may bypass the action of the lysosomes by their active penetration into the cytoplasm and avoidance of the vacuoles.

In the case of non-activated monocytes being used to propagate rickettsiae as is the case in the present study, one must assume that these cells possess the necessary cell surface receptors needed for attachment and entry of the organisms (Silverstein, 1977). In this circumstance, however, some participation by the organism in the form of energy-yielding metabolism as proposed by Weiss (1973) must be needed for its entry.

While the question regarding mechanisms whereby cultured monocytes serve as an efficient host for propagation of a variety of rickettsiae must await further studies, the practical advantage of the method is obvious. There is no rapid and

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Weiss, Emilio: Growth and Physiology of Rickettsiae. *Bact. Rev.*, (1973): 259-283.

Silverstein, C. C.: Endocytic Uptake of Particles by Mononuclear Phagocytes and the Penetration of Obligate Intracellular Parasites. *Am J Trop Med Hyg*, 26 (1977): 161-168.

simple technique that may be used in substantiating clinical evidence of a rickettsial disease by isolation of the causative agent. Isolation of the organism in laboratory animals such as mice may require weeks and sometimes months. As with other rickettsiae described in this communication, isolation and cultivation of peripheral blood monocytes from suspected sennetsu rickettsiosis patients may prove to be an early and accurate diagnostic means.

Sequences of the cycle of development of R. sennetsu in human monocytes greatly resemble similar development of E. canis in canine monocytes (Nyindo et al, 1971). Three developmental forms, elementary bodies (single organism), small inclusion bodies (initial bodies), and large inclusion bodies (morulae) described with E. canis were also noted with R. sennetsu. The question, whether this morphologic resemblance of growth stages between E. canis and R. sennetsu is a real reflection of their close biological relationship or if it is merely a morphologic expression forced upon the organism by a common host cell (monocyte) must await further studies.

5. Summary: Rickettsia sennetsu, the causative agent of sennetsu rickettsiosis has been propagated in monocyte cell cultures derived from apparently normal human beings. Tissue culture medium consisted of Eagle's minimum essential medium supplemented with 10% human serum. Microscopic examination of Giemsa stained specimens of monocytes derived from the supernatant of cultures suggest that intracytoplasmic R. sennetsu underwent a specific cycle of development. Principal developmental forms

of the organism in the sequence of their appearance during 20 days of incubation were loosely scattered individual organisms, organismal clusters with individual rickettsiae easily differentiated, small and large inclusion bodies with undifferentiated individual organisms, individual organism and inclusion bodies in large cytoplasmic vacuoles, and various cell-free organismal growth forms in close proximity to disintegrated monocytes.

This development sequence appears similar to that of E. canis propagated in canine monocytes. Specific identification of R. sennetsu was made by staining cultured organisms with fluorescein-conjugated globulins extracted from pooled sera of patients convalescing from the disease. Mice inoculated with the cultured organism developed gross-pathologic changes indicative of infection and the organism was demonstrated in their spleens, peritoneal macrophages and mononuclear blood cells.

Based upon earlier experiences with monocyte cell cultures, it appears that the method may be useful for an early diagnosis of sennetsu rickettsiosis by isolation of the organism from the blood of affected individuals.

ADDENDUM: Serodiagnostic services in support of control of blood diseases of military dogs.

During the past year of support this laboratory, in addition to conducting research under the 3 objectives as described above, has continued with serodiagnostic services in support of the U.S. Armed Forces (Army and Air Force) aimed at control of hemotropic diseases of military dogs. These sera have been referred to us by way of LTC Edward H. Stephenson of the Walter Reed Army Institute of Research.

- A. Serodiagnosis of canine ehrlichiosis - tropical canine pancytopenia by use of the indirect fluorescent antibody test (IFA)
  - 1. U.S. Armed Forces dogs stationed in the continental United States - 146 sera or 418 tests.
  - 2. U.S. Air Force dogs from various bases abroad - 297 sera or 882 tests.
  - 3. U.S. and Malaysian Army dogs - 592 sera or 1042 tests.
- B. Serodiagnosis of canine babesiosis - using indirect fluorescent antibody (IFA) test:
  - 1. U.S. Armed Forces dogs stationed in continental United States - 48 sera or 480 tests.
  - 2. U.S. and Malaysian Army dogs - 148 sera or 888 tests.

Total sera 1231 - total tests 3710.

Upon instruction from the Office of Research Management of the Walter Reed Army Institute of Research, WRAMC, a separate contract proposal entitled, "Serodiagnostic Services in Support of Field Operations of U.S. Armed Forces and its Canine Corps" was submitted for consideration during the past year. Approval of this contract would enable more efficient utilization of funds toward proposed research.

### III. PUBLICATIONS PRODUCED DURING THE PAST YEAR OF SUPPORT

Lewis, G. E. Jr., Sheryl Hill, Miodrag Ristic: Effect of Canine Immune Serum on the Growth of Ehrlichia canis within Nonimmune Canine Macrophages. Am J Vet Res, 39 (1) (Jan. 1978): 71-76.

Nyindo, M. B. A., Miodrag Ristic, G. E. Lewis, Jr., D. L. Huxsoll, Edward H. Stephenson: Immune Response of Ponies to Experimental Infection with Ehrlichia equi. Am J Vet Res., 39 (1) (Jan. 1978): 15-18.

Lewis, G. E. Jr. and Miodrag Ristic: Effect of Canine Immune Macrophages and Canine Immune Serum on the Growth of Ehrlichia canis. Am J Vet Res, 39 (1) (Jan. 1978): 77-82.

Montrey, R. D., D. L. Huxsoll, S. Arimbalam, and M. Ristic: Experimental Infection of Mixed Breed Dogs with Ehrlichia canis. Mal Vet J, 6 (146) (1977): 146-156.

Kakoma, I., C. A. Carson, M. Ristic, E. H. Stephenson, P. K. Hildebrandt, D. L. Huxsoll: Platelet Migration Inhibition as an Indicator of Immunologically Mediated Target Cell Injury in Canine Ehrlichiosis. Infect Immun 20 (1) (Apr. 1978): 242-247.

Smith, R. D., D. M. Sells, G. E. Lewis, Jr. and M. Ristic: Development and Maintenance of Ehrlichia canis in its Invertebrate and Vertebrate Hosts. Conf on Tick-Borne Diseases and their Vectors, Edinburgh, Scotland, Sept. 26-Oct 1, 1976 (1978): 517-518.

Ristic, Miodrag: Tick-Borne Rickettsias of Veterinary Importance with Emphasis on the Immunology of the Disease. Conf on Tick-Borne Diseases and their Vectors, Edinburgh, Scotland, Sept 26-Oct 1, 1976 (1978): 475-481.

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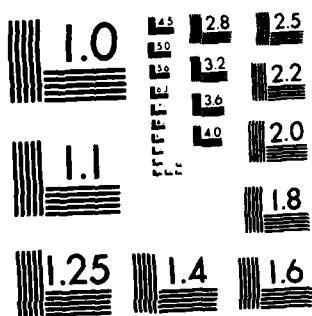
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- Davidson, D. E., Jr., Garrett S. Dill, Jr., M. Tingpalapong, S. Premabutra, P. La-or Nguen, E. H. Stephenson, M. Ristic: Prophylactic and Therapeutic Use of Tetracycline During an Epizootic of Ehrlichiosis Among Military Dogs. JAVMA 172 (6) (Mar 1978): 697-700.
- Ristic, Miodrag, and C. A. Carson: Humoral and Cell-Mediated Immune Responses to Ehrlichia canis and Ehrlichia equi. Conf on Tick-Borne Diseases and their Vectors, Edinburgh, Scotland, Sept 26-Oct 1, 1976 (1978): 523-526.
- Ristic, M., D. L. Huxsoll, H. Tachibana, G. Rapmund: Serologic Relationship Between Causative Agents of Human Sennetsu Rickettsiosis and Canine Ehrlichiosis (in preparation).

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